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THE ROLE OF SYNAPTIC MICRORNA IN CHRONIC ALCOHOL CONSUMPTION AND ITS EFFECTS ON SYNAPTIC COMPOSITION

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BY

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I DEDICATE THIS TO MY PARENTS PNINA YOUNG, BRAD YOUNG AND MIKE MOST.
I DERIVE MY INSPIRATION FROM YOU.

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THE ROLE OF SYNAPTIC MICRORNAS IN CHRONIC ALCOHOL CONSUMPTION AND ITS EFFECTS ON SYNAPTIC COMPOSITION

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Drug dependence is the process by which the brain learns to depend on a drug and crave for it in its absence. This learning takes place through modifications of synaptic connections between neurons, changing synaptic structure and function, and causing long-lasting neuroadaptations. Over the past decade, several microRNAs have been proposed as playing a key role in regulating local mRNA translation into protein, specifically in the synaptic compartments of the cell (pre-synaptic terminals and post-synaptic densities). There is limited evidence, however, regarding how synaptic microRNAs control local mRNA translation during chronic drug exposure and how this contributes to the development of dependence. Can alcohol-responsive synaptic mRNAs and microRNA be identified? Do synaptic microRNAs regulate the synaptic mRNA expression changes in response to alcohol? Can changes in synaptic microRNA composition affect alcohol consumption?

In this thesis, I present research supporting microRNA regulation of local mRNA translation and how drugs of abuse target this process. In the first section, I focus on the identification of alcohol-responsive synaptic mRNAs. In the second section, I focus on identifying alcohol-responsive synaptic microRNAs and predict key mRNA-microRNA

interactions. Identifying the primary regulatory elements in the synapse is imperative for an accurate model of the synaptic interactions that lead to mRNA translation underlying plasticity. Since the neuroadaptations associated with response to alcohol rely on many mRNAs, therapy with microRNAs provides a potential treatment for alcohol dependence. In the third section, I focus on manipulating key microRNAs in-vivo to reverse and prevent alcohol consumption and alcohol-induced-neuroadaptations. The ability of synaptic microRNAs to rapidly regulate mRNAs provides a discrete, localized system that could potentially be used as diagnostic and treatment tools for alcohol and other addiction disorders.

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CHAPTER 1: INTRODUCTION

Overview

The first chapter of this thesis consists of two main sections. In the first, I review the effects of alcohol consumption on the central nervous system as a whole. In the second, I focus specifically on the synaptic compartment of the cell, and detail the interplay between alcohol consumption, synaptic mRNAs and microRNAs.

The work presented in the first section of this chapter has been published as a review in 2014 in the Handbook for Clinical Neurology. Most D, Ferguson L, Harris RA. Molecular basis of alcoholism. 125:89-111. PubMed PMID: 25307570. The published text has been modified in order to fit the thesis.

The work presented in the second section of this chapter has been published as a review in 2014 in Frontiers Molecular Neuroscience. Most D, Workman E, Harris RA. Synaptic adaptations by alcohol and drugs of abuse: changes in microRNA expression and mRNA regulation. 7:85. PubMed PMID: 25565954. The published text has been modified in order to include some more recent information which is pertinent to this thesis.

The Effects of Alcohol on the Nervous System

Alcohols are organic compounds containing a hydroxyl (-OH) group attached to a carbon atom and the aliphatic alcohols are described by the general formula $C_nH_{2n+1}OH$. Ethanol, the psychoactive constituent of alcohol, has been used recreationally for tens of thousands of years (Hanson 1995) and is one of the largest health burdens on society (Cargiulo 2007). For the remainder of this thesis, alcohol will refer to ethanol, and alcoholism as the functional equivalent of alcohol use disorder, as outlined by the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-V) (American Psychiatric Association, 2013).

The maladaptive behaviors of alcoholism rely on changes in the brain that lead to compulsive and excessive drinking, afflicting all organs, with damage as a secondary

consequence of alcoholism. While acute use of alcohol, such as binge drinking and intoxication, causes cellular changes in the brain that last for hours, chronic alcohol use induces widespread neuroadaptations in the nervous system that can last a lifetime. This involves the remodeling of synapses that are dependent upon changes in gene expression in the presence of chronic alcohol use (Wilke, Sganga et al. 1994), and is illustrated in Figure 1.1.

In order to fully understand the effects of alcohol on behavior and thus enable the development of efficacious treatments, it is necessary to understand the actions of alcohol at the molecular level. It is remarkable how little is known about alcohol's molecular targets in view of alcohol's burden on public health and its long-term and widespread use. One reason for this might be alcohol's low binding affinity to proteins, reflected by the fact that clinically relevant intoxication levels of alcohol are measured in millimolar concentrations whereas most other drugs of abuse are measured in nanomolar concentrations. A consequence of the high concentrations in the body is that there are a large number of potential molecular targets.

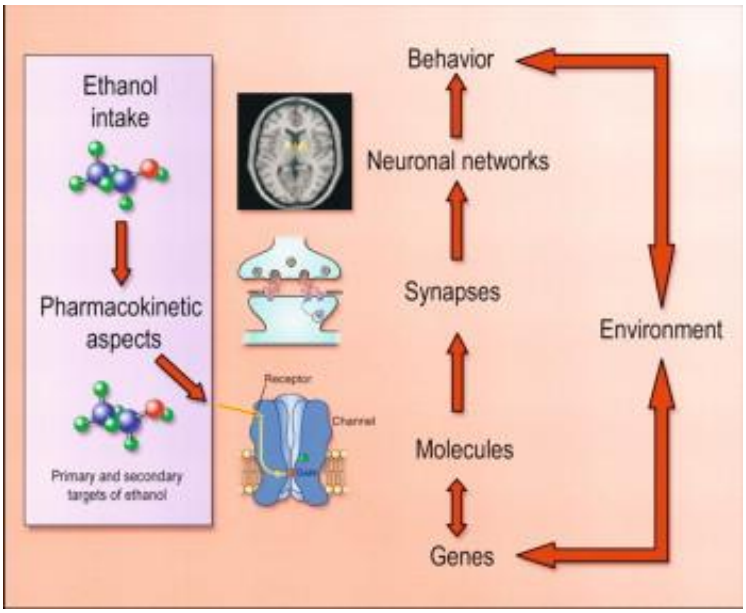


FIGURE 1.1: A SCHEMATIC ILLUSTRATING THE DYNAMIC INTERACTIONS BETWEEN ALCOHOL AND THE NERVOUS SYSTEM

Figure has been adapted from Spanagel, 2009.

In the early days of alcohol research, it was thought that because of the small size and non-specific nature of the alcohol molecule, it would likely not have a specific binding domain on proteins, but would instead interfere with the lipid membranes of the central nervous system (CNS). However, alcohol has only been shown to interact with the lipid bilayer at concentrations much higher than clinically relevant (Pang, Braswell et al. 1980, Goldstein 1984, Peoples, Chaoying et al. 1996). For this reason, researchers have focused their search on other molecular targets of alcohol. Most of the examples below come from postmortem tissue analysis of alcoholics, animal models, and cell cultures. Many of the molecular pathways that are sensitive to alcohol are highly conserved across species such as humans, mice, rats, worms, and fruit flies (Dick and Foroud 2002, Mayfield, Lewohl et al. 2002). In addition, considerable progress has been made in defining binding

cavities for alcohol in several proteins, including ion channels (Harris, Trudell et al. 2008, Howard, Slesinger et al. 2011, Sauguet, Howard et al. 2013).

This section of the chapter will discuss the known and proposed molecular targets of alcohol in the brain that may be important for behaviors meeting the DSM-V criteria for alcohol use disorder. Additionally, the molecular mechanisms of approved and prospective treatments for alcoholism will be discussed.

Primary Targets

Alcohol's effects on the brain are diverse and include changes in levels and function of neurotransmitters, synaptic changes in brain circuitry regulating compulsivity and inhibition. Changes in these molecular systems lead to tolerance and withdrawal when alcohol is removed from the system.

Alcohol metabolism and distribution can be summarized as follows: After consumption, alcohol is absorbed into the blood stream through the stomach and intestines where it readily crosses the blood–brain barrier. Alcohol is then distributed with body water and is found at approximately the same concentration in all tissues, including the nervous system. Most of the metabolism of alcohol occurs in the liver, whereas the brain has limited metabolism by the mitochondrial cytochrome P450 (CYP2E1), catalase, and other pathways (Zakhari 2006). See Table 1.1 for a list of the alcohol-target genes discussed in this chapter.

Gene symbol	Gene name	Function	Reference
CYP2E1	Cytochrome P450	Involved in alcohol metabolism.	(Zakhari 2006)
ADH	Alcohol dehydrogenase	Involved in alcohol metabolism. Binds alcohol through a zinc atom on ADH and the hydroxyl group of alcohol.	(Zakhari 2006)
CBP	CREB-binding protein	Allows for transcriptional activation through histone acetylase activity. Acute alcohol exposure increases levels of CBP. Withdrawal produces the opposite effect.	(Pandey, Ugale et al. 2008)
NPY	Neuropeptide Y	Acute alcohol exposure increases levels of NPY. Withdrawal produces the opposite effect.	(Pandey, Ugale et al. 2008)
HDAC5	Histone deacetylase 5	Mice lacking HDAC5 become hypersensitive when chronically exposed to cocaine. Chronic cocaine administration inactivates HDAC5 by exporting it out of the nucleus, resulting in histone hyperacetylation and increased mRNA expression of HDAC5 target genes.	(Renthal, Maze et al. 2007)
NK1R	Neurokinin 1 receptor	NK1R is increased during both chronic cocaine and alcohol exposure. Silencing the translation of the NK1R using RNA interference was found to reduce alcohol consumption in mice. NK1R antagonist (L822429) decreases voluntary alcohol consumption, suppresses stress-induced reinstatement of alcohol seeking, and increases sensitivity to the sedative effects of alcohol in rats. An NK1R antagonist suppresses spontaneous alcohol cravings, improves overall well-being, blunts cravings induced by a challenge procedure, and attenuates concomitant cortisol responses in detoxified alcoholic inpatients. An analysis of brain function of human alcoholics performing behavioral emotional tasks, corroborates the above results, suggesting NK1R antagonism as a potential therapeutic target for the treatment of alcoholism.	(George, Gilman et al. 2008, Baek, Jung et al. 2010, Schank, Pickens et al. 2011)

TABLE 1.1: SUMMARY OF GENES DISCUSSED IN THE FIRST SECTION OF THIS CHAPTER

CYP2E1 metabolism of alcohol produces acetaldehyde and is a source of acetaldehyde in the brain. Additional acetaldehyde may enter the brain from peripheral conversion of alcohol to acetaldehyde by an enzyme found in the liver called alcohol dehydrogenase (ADH). In fact, ADH can be considered a "target" of alcohol since it binds alcohol through a zinc atom on ADH and the hydroxyl group of alcohol.

In the periphery, acetaldehyde is the primary metabolite of alcohol and is responsible for the flushing effect that encompasses face flushing, nausea, vomiting, headache, tachycardia, and sweating. Disulfiram (Antabuse), the first of three approved

treatments for alcoholism, employs the aversive nature of acetaldehyde by inhibiting aldehyde dehydrogenase enzyme, thereby allowing for an accumulation of acetaldehyde. See Table 1.2 for a list of the pharmaceutical treatments discussed in this chapter.

Drug	Description	Advantages	Disadvantages
Disulfiram (Antabuse)*	Inhibitor of aldehyde dehydrogenase enzyme.	Prevents alcohol consumption.	Aversive therapy and does not reduce alcohol cravings, leading to poor patient compliance.
Acamprosate (Campral)*	Mechanism of action is unclear. Proposed to be altering glutamatergic system.	Few side-effects and high patient compliance.	Treatment is available only for patients who had already been withdrawn from alcohol. Abstinence rates are lower than naltrexone and the combination of naltrexone and acamprosate.
Naltrexone*	Mu-opioid receptor antagonist.	Reduces craving. Highest abstinent rates among FDA-approved drugs for alcoholism.	Anxiety, trouble sleeping and nausea. A better response to naltrexone has been associated with possession of the G allele of the A118G polymorphism of the mu-opioid receptor gene (OPRM1).
Benzodiazepines#	GABA-A positive modulators.	Treats withdrawal symptoms.	Has abuse potential. Causes sedation. Causes cross-tolerance with alcohol.
Ondansetron (Zofran)#	5-HT3 receptor antagonist.	May reduce alcohol consumption.	
Fluoxetine (Prozac)#	Selective serotonin reuptake inhibitor (SSRI).	May reduce alcohol consumption in some patients (late-onset individuals).	Human studies show inconsistent results. SSRI side effects.
Fluphenazine#	Dopamine receptor antagonist.	Reduced alcohol consumption in rodents.	No clinical trials have been performed. Typical antipsychotic side-effects.
Aripiprazole (Abilify)#	Dopamine receptor antagonist.	Reduces craving and is well tolerated.	Atypical antipsychotic side-effects.
Topiramate (Topamax)#	Mechanism of action is unclear. Associated with glutamatergic system inhibition.	Successful in treating heavy drinkers.	May effect memory/thinking and cause sedation.
Baclofen#	GABA-B agonist		Efficacy is controversial
Pioglitazone (Actos)#, Rosiglitazone (Avandia)#, Clofibrate (Atromid-S)#	Peroxisome proliferator activator receptor (PPAR) γ-agonists (pioglitazone and rosiglitazone)	Drugs have been found to be hepatoprotective and neuroprotective in rodents	No published human data
Minocycline (Minocin)#, Doxycycline#, Anakinra	Anti-inflammatory antibiotics	Well tolerated	No human data. Alters metabolism of oral contraceptives

Drug	Description	Advantages	Disadvantages
(Kineret)#, Indomethacin (Indocin)#, CAPE			
Gabapentin#	GABA analog, calcium channel and GABA modulator	Increases sleep quality in alcoholics. Analgesic and anxiolytic	Causes sedation. Does not affect mood or craving

TABLE 1.2: SUMMARY OF PHARMACEUTICAL TREATMENTS DISCUSSED IN THE FIRST SECTION OF THIS CHAPTER

*Food and Drug Administration (FDA)-approved for treatments of alcoholism. #FDA-approved for the treatment of other diseases. Baclofen refers to Kemstro, Lioresal, Liofen, Gablofen, Beklo and Baclosan. Doxycycline refers to Vibramycin, Monodox, Oracea, Doryx, Vibrox, Adoxa, Doxyhexal, Doxylin, Doxoral, Doxy-1 and Atridox. GABA (γ-aminobutyric acid).

Disulfiram is a rather non-specific enzyme inhibitor and may be useful in the treatment of cocaine dependence and even cancer chemotherapy due to actions on sites other than aldehyde dehydrogenase (Shorter and Kosten 2011, Schmitt, Frezza et al. 2012). Although disulfiram decreases drinking when taken regularly, it has low patient compliance because of its aversive effects (Moriarty 1950, Barth and Malcolm 2010). In addition to the peripheral flushing effect, acetaldehyde may have actions in the brain relevant to the acute effects of alcohol (Quertemont, Tambour et al. 2005) and even on the development of alcoholism (Deng and Deitrich 2008). Acetaldehyde is self-administered by rodents through intravenous and intracerebral ventricular routes (Amit, Brown et al. 1977, Myers, Ng et al. 1984). Rats show preference for the physical place where they received central or peripheral administration of acetaldehyde over places where they received saline (conditioned place preference, abbreviated as CPP) (Quintanilla and Tampier 2003, Spina, Longoni et al. 2010). Although more research is necessary to fully understand these mechanisms, the neurobiological – and consequently the behavioral – actions of alcohol most likely depend on central contributions from both alcohol and its metabolites, acetaldehyde and acetate (Jiang, Gulanski et al. 2013). The development of alcohol tolerance and dependence comes from alterations in brain

structure and function over time, and is long-lasting. This involves the remodeling of synapses that are dependent upon changes in gene expression in the presence of chronic alcohol (Rhodes and Crabbe 2005). Here we focus on the molecular adaptations at the acute and chronic stages of alcohol use that underlie the hallmarks of alcoholism: withdrawal, tolerance, relapse, and craving.

It is important to note that these long-lasting drug responsive alterations are unlikely to be encoded in RNAs or proteins due to the fast turnover rate of those molecules. This suggests that DNA is in charge of cellular memory, and that epigenetic mechanisms may be critical components of long-term learning and memory processes (Levenson and Sweatt 2005, Ponomarev, Wang et al. 2012), as well as chronic dependence on drugs (Renthal and Nestler 2008).

THE EFFECTS ON DNA

Both the sequence and structure of DNA molecules control all downstream processes such as RNA transcription and protein translation, and both can contribute to the development, progression, and persistence of alcoholism. Recent research has emphasized the notion that epigenetic mechanisms (which exert lasting control over gene expression through structural modifications of the DNA without altering the sequence) could mediate stable changes in brain function associated with alcoholism (Wallner, Hanchar et al. 2003). DNA methylation, histone acetylation, and phosphorylation are three common epigenetic-enzymatic modifications to chromatin structure that make the DNA more or less accessible to transcription factors and enzymes, thus changing the transcriptional activity of the target genes. Chronic exposure to alcohol was found to induce changes in the chromatin structure, specifically on gene promoters causing changes in gene expression in alcoholics (Guerri and Pascual 2010, Ponomarev, Wang et

al. 2012). This suggests that epigenetic mechanisms are involved in both biochemical and behavioral responses to alcohol.

Histone deacetylase (HDAC) is an enzyme that removes the acetyl group from histone proteins on DNA, making the DNA less accessible to transcription factors. Acute alcohol was found to decrease HDAC activity and increase acetylation of histones (H3 and H4) in the rat amygdala. Conversely, withdrawal from chronic alcohol in rats was found to increase HDAC activity and decrease H3 and H4 acetylation in the rat amygdala (Pandey, Ugale et al. 2008). An HDAC inhibitor (trichostatin A) blocks the increase in HDAC activity and rescues the deficits in H3 and H4 acetylation in the amygdala. This change prevents the development of withdrawal-related anxiety in rats, suggesting a potential role for HDAC inhibitors as therapeutic agents in treating alcohol withdrawal symptoms (Pandey, Ugale et al. 2008). CREB-binding protein (CBP) is a histone acetylase (HAT) that acetylates nearby histones, and allows for subsequent transcriptional activation. Acute alcohol exposure increases levels of CBP and neuropeptide Y (NPY), while withdrawal produces the opposite effect. Moreover, the withdrawal-induced anxiety behavior was found to correlate with the levels of CBP and NPY in the amygdala (Pandey, Ugale et al. 2008). Mutations in CBP cause a form of mental retardation in humans (Tsankova, Renthal et al. 2007), suggesting it is important for normal learning and memory mechanisms in the brain. This finding goes hand in hand with the well-known hypothesis that alcoholism is an aberrant process of learning and memory (Hyman, Malenka et al. 2006).

An example of a target gene of HDAC5 that is found to be increased during both chronic cocaine and alcohol exposure is neurokinin 1 receptor (NK1R). This receptor is also known as the substance P receptor due to its importance in pain transmission. Silencing the translation of the NK1R using RNA interference reduces alcohol drinking in mice, emphasizing the role that NK1R plays in alcoholism (Baek, Jung et al. 2010). In fact,

an NK1R antagonist (L822429) decreased voluntary alcohol consumption, suppressed stress-induced reinstatement of alcohol seeking, and increased sensitivity to the sedative effects of alcohol in rats (Schank, Pickens et al. 2011). Similar effects were also seen in detoxified alcoholic inpatients, as an NK1R antagonist suppressed spontaneous alcohol cravings, improved overall well-being, blunted cravings induced by a challenge procedure, and attenuated concomitant cortisol responses. Furthermore, functional magnetic resonance imaging (fMRI) of brains of human alcoholics, during a behavioral emotional task was performed and corroborated the above results, suggesting NK1R antagonism as a potential therapeutic target for the treatment of alcoholism (George, Gilman et al. 2008). Figure 1.2 summarizes the epigenetic changes associated with alcohol.

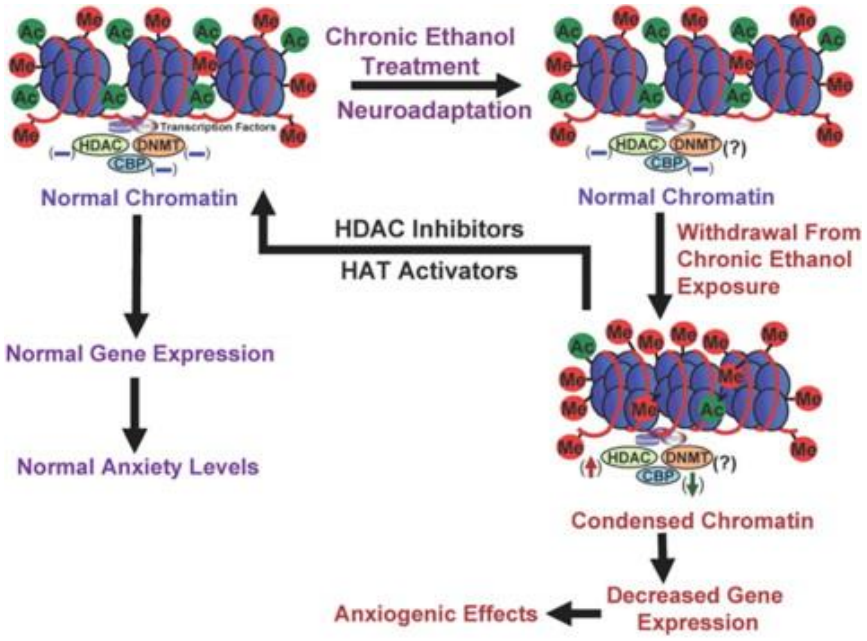
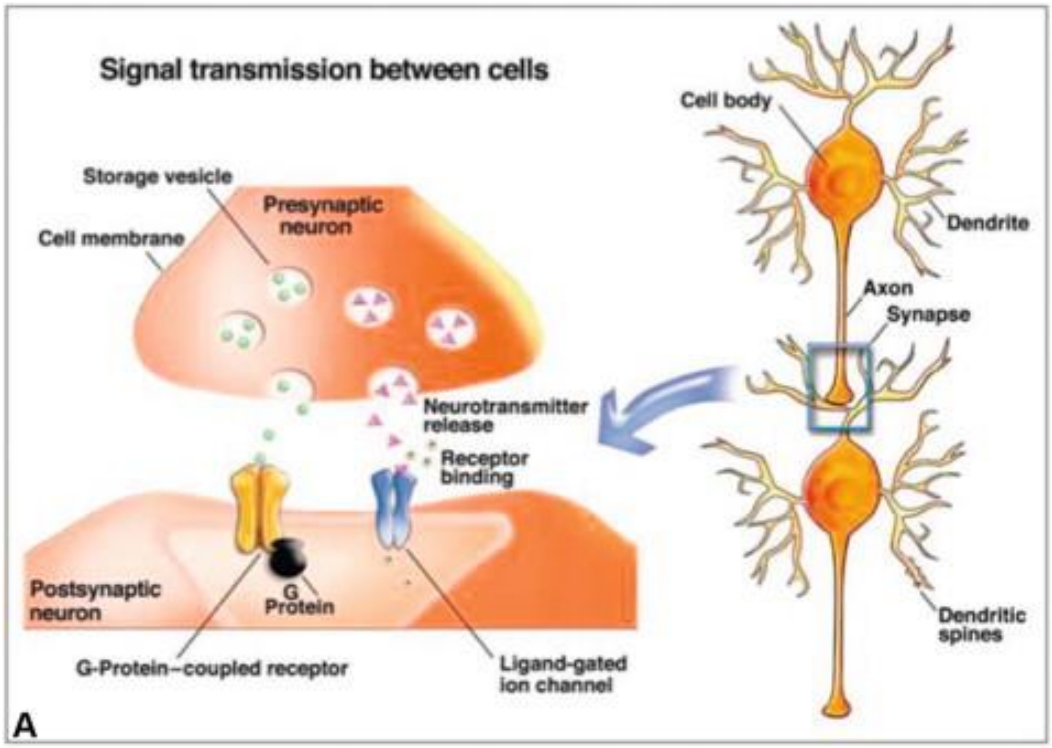


FIGURE 1.2: EPIGENETIC MODIFICATIONS ASSOCIATED WITH ALCOHOLISM

CREB-binding protein (CBP) and histone deacetylase (HDAC) activity are altered by acute alcohol exposure. Chronic alcohol exposure results in neuroadaptations opposing the acute effects of alcohol in order to maintain a homeostatic state. Withdrawal after chronic alcohol exposure is associated with increased HDAC activity and decreased levels of CBP and the associated histone acetylation. DNMT, DNA methyltransferase; Ac, acetyl; Me, methyl; HAT, histone acetylase. Figure has been adapted from Starkman et al., 2012.

DNA is the master regulator in the cell and is possibly a molecular implementation of the persistence of drug effects (and even memories) in the brain. DNA exerts its control through gene expression which includes: RNA transcription, protein translation, and regulatory processes like microRNA and RNA splicing. In addition to alcohol's indirect effect on gene expression via epigenetic actions on chromatin, alcohol can directly target the gene expression machinery, which will be explored below and is detailed in Figure 1.3.



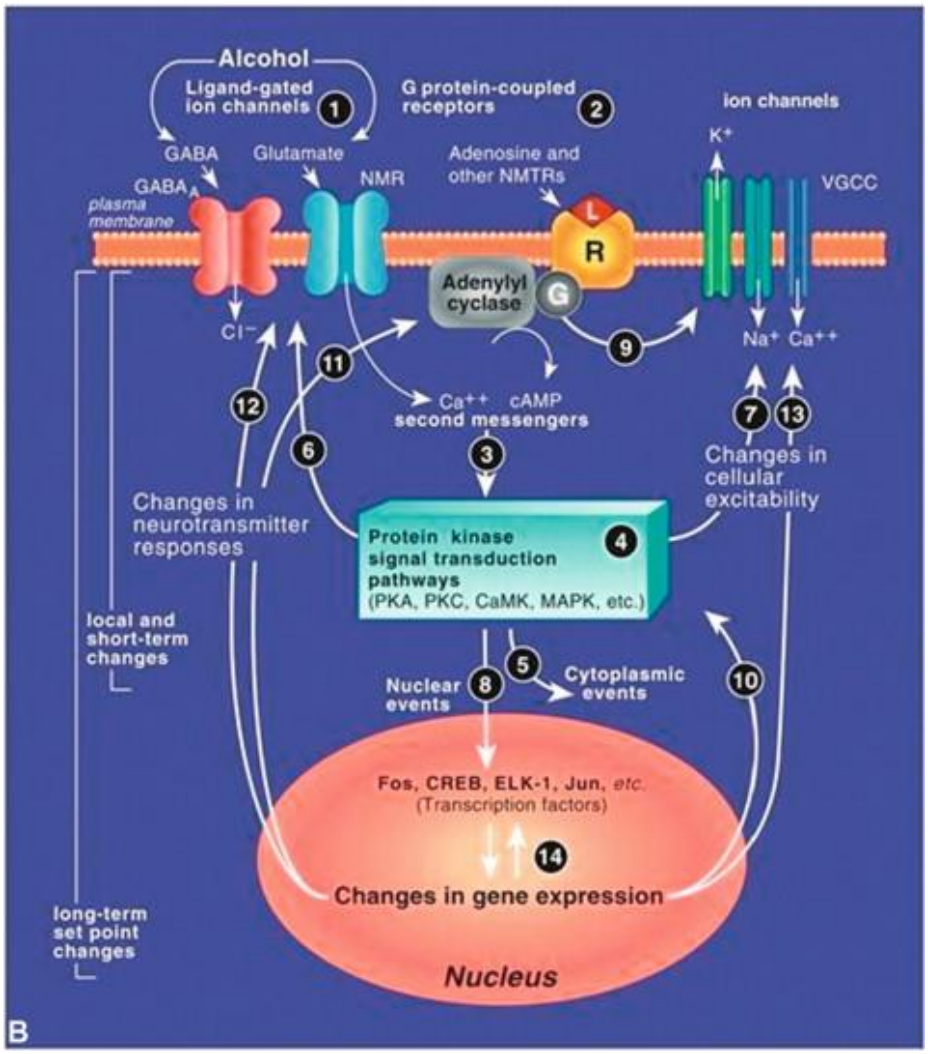


FIGURE 1.3: THE 'NORMAL' SYNAPSE AND THE CELLULAR ADAPTATIONS IN RESPONSE TO CHRONIC ALCOHOL CONSUMPTION

A. Synaptic transmission is the process by which the brain communicates information. Depending on the inputs to the presynaptic neuron's dendrites, an action potential will be generated at the axon hillock and propagate down the axon through the movement of charged particles (i.e., the ions – Na+ and K+). The synapse is the gap between the axon of the presynaptic neuron and the dendrite of the postsynaptic neuron. In order for the information to cross the synapse, the electrical signal of the action potential must be converted into a chemical signal. This is achieved by releasing neurotransmitters from the presynaptic nerve terminal in a voltage-dependent (and therefore calcium-dependent) manner. The presynaptic nerve terminal contains the neurotransmitter release machinery needed for this to occur. Once released, the neurotransmitter will diffuse across the synapse and bind to receptors on the postsynaptic nerve's dendrites. The two major types of receptors of concern here are ligand-gated ion channels and G-protein-coupled receptors. Ligand-gated ion channels undergo a conformational change when a ligand (i.e., neurotransmitter) is bound and allow a particular ion (e.g. Cl–, Na+, Ca2+ , etc.) to flow into or out of the cell. When G-protein-coupled receptors are activated, they affect secondary messengers and molecular cascades, resulting in changes in the postsynaptic neuron. It is necessary to understand the basics of synaptic transmission because acute

and chronic alcohol exposure cause changes in many molecules important for transmission as well as changes transmission properties. Figure has been adapted from Clapp et al., 2008. B. An example of a synapse stimulated by alcohol use and the molecular effects on cellular cascades. VGCC, voltage-gated calcium channel; CaMK, calmoldulin kinase; MAPK, mitogen-activated protein kinase. Figure has been adapted from Koob et al., 2005.

THE EFFECTS ON NEUROTRANSMITTER SYSTEMS

Glutamate

Glutamate is the primary excitatory neurotransmitter in the brain. Glutamatergic (glutamate-using) systems contain both ionotropic ligand-gated receptors coupled to the flow of charged particles (NMDA, AMPA and Kainate) and metabotropic (mGluR) receptors. The glutamatergic system plays an important role in long-term potentiation and long-term depression and therefore has physiological implications that are important for learning and memory. Generally, all glutamate receptors are inhibited by acute alcohol treatment, although some subtypes are only affected by very high concentrations. Alcohol's acute actions on the glutamatergic system have been implicated in tolerance, withdrawal, craving, relapse, and dependence. Alcohol acts as a non-competitive inhibitor of the AMPA/kainate receptors at high concentrations (Dildy-Mayfield and Harris 1992, Dildy-Mayfield and Harris 1995, Akinshola, Yasuda et al. 2003). Out of all the glutamatergic receptors, the NMDA receptor (NMDAR) is highly sensitive to the effects of alcohol. Alcohol's binding site on the NMDAR is not known, and there has been some evidence to suggest that alcohol exerts its effect on the NMDAR through protein kinase C (Li, Mochly-Rosen et al. 2005). Acute application of alcohol to hippocampal neuronal slices reduces NMDA activity at a concentration that produces intoxication in humans and is linearly related to alcohol's intoxicating potency (Lovinger, White et al. 1989). In a drug discrimination test, MK-801, an NMDA antagonist, elicited the same response that animals were trained to give in the presence of alcohol, indicating that alcohol's NMDA-antagonizing effects are important for mediating the subjective effects of alcohol

(Butelman, Baron et al. 1993). In recently detoxified alcoholics, ketamine, an NMDA antagonist, was able to mimic the behavioral effects of alcohol (Krystal, Petrakis et al. 1998). Ketamine reduces alcohol consumption in alcohol-preferring rats (dose-dependently), and this mechanism is mediated through the mammalian target of rapamycin (mTOR) pathway (Sabino, Narayan et al. 2013). Ketamine has also been found effective in treating major depression disorder (Rasmussen, Lineberry et al. 2013) as well as depressive symptoms, such as the depressive-like behavior produced by abstinence from alcohol (Holleran, Wilson et al. 2016). When tested on individuals with a family history of alcohol dependence, ketamine caused an attenuated response in dysphoric mood, relative to those without such a family history (Petrakis, Limoncelli et al. 2014). The production of alcohol-like effects by ketamine and the reduction in alcohol consumption and alcohol-related symptoms supports the clinical role of NMDAR function facilitating alcohol's effects on humans.

The response to alcohol depends on the subunit composition of the glutamatergic receptor. A study using rat NMDAR subunits expressed in Xenopus oocytes suggests that NMDARs containing certain subunits are more sensitive to alcohol than others (Raeder, Holter et al. 2008). Alterations in subunit composition of glutamate receptors were seen after chronic use of alcohol in mice (Ortiz, Fitzgerald et al. 1995). Chronic alcohol was also found to increase the expression of AMPA receptor (AMPAR) subunit (GluA) 1 in the brains of alcoholics (Lewohl, Wang et al. 2000).

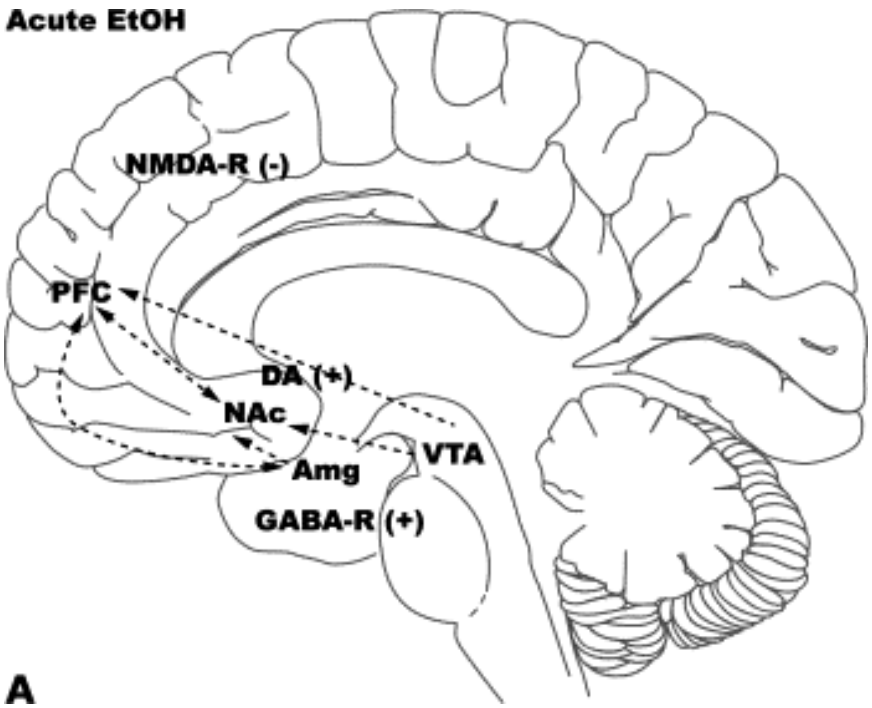
Once alcohol is removed from the system, the hyperglutamatergic state can produce a severe withdrawal syndrome characterized by agitation, anxiety, and disorientation and is associated with a susceptibility to seizures and excitotoxic cell death. NMDAR antagonists can be applied to protect cells from this type of death (Hoffman, Lorio et al. 1995, Al Qatari, Khan et al. 2001). The acute withdrawal syndrome is clinically

relevant and well characterized. The question remains as to whether the neuroadaptations leading to acute withdrawal syndrome contribute to the propensity of relapse or lead to alcoholism.

Acute alcohol inhibits the function of the NMDAR, while chronic use of alcohol seems to upregulate NMDAR expression in the brain (Qiang and Ticku 2005). This upregulation is thought to counteract the acute inhibition of the glutamatergic system and also the sedative effects of increased g-aminobutyric acid receptor A (GABA-A) activity, as discussed below.

Acamprosate, one of the three approved treatments for alcoholism, is proposed to exert at least part of its effect by altering glutamatergic function (De Witte, Littleton et al. 2005), though the exact mechanism by which it interacts is unclear. Acamprosate was found to reduce relapse rate, increase abstinence rate, and decrease excessive drinking in alcohol-dependent rats, and had no effect in non-dependent rats (Spanagel, Hölter et al. 1996, Spanagel, Putzke et al. 1996, Spanagel, Zieglgänsberger et al. 1996). In dozens of clinical trials conducted in Europe, about half of the alcoholics treated with acamprosate maintained sobriety compared to the placebo group (Mason and Ownby 2000, Mann, Lehert et al. 2004), but results of US clinical trials have shown less beneficial effects of acamprosate (Anton, O'Malley et al. 2006, Mason, Goodman et al. 2006). Several studies seeking to identify acamprosate's mechanism of action have failed to show direct modulation of NMDARs at clinically relevant concentrations (Reilly, Lobo et al. 2008) and a recent study suggests that calcium is the active moiety of acamprosate (Spanagel, Vengeliene et al. 2014). Application of acamprosate on GABA-A receptors and voltage-gated Na^+ channels did not exert any effect (Reilly, Lobo et al. 2008). On the other hand, knocking out some of the subunits of the mGluRs in mice or by using an mGluR antagonist reduces the ability of acamprosate to affect behavior (Blednov and Harris 2008). Taken

together, the evidence indicates that alcohol's modulation of the glutamatergic system is an important molecular mechanism by which alcohol exerts its behavioral effects and a potential target for the treatment of alcoholism. Figure 1.4 presents an illustration of the main neurotransmitter systems and brain regions involved in the neuroadaptations associated with acute and chronic alcohol use.



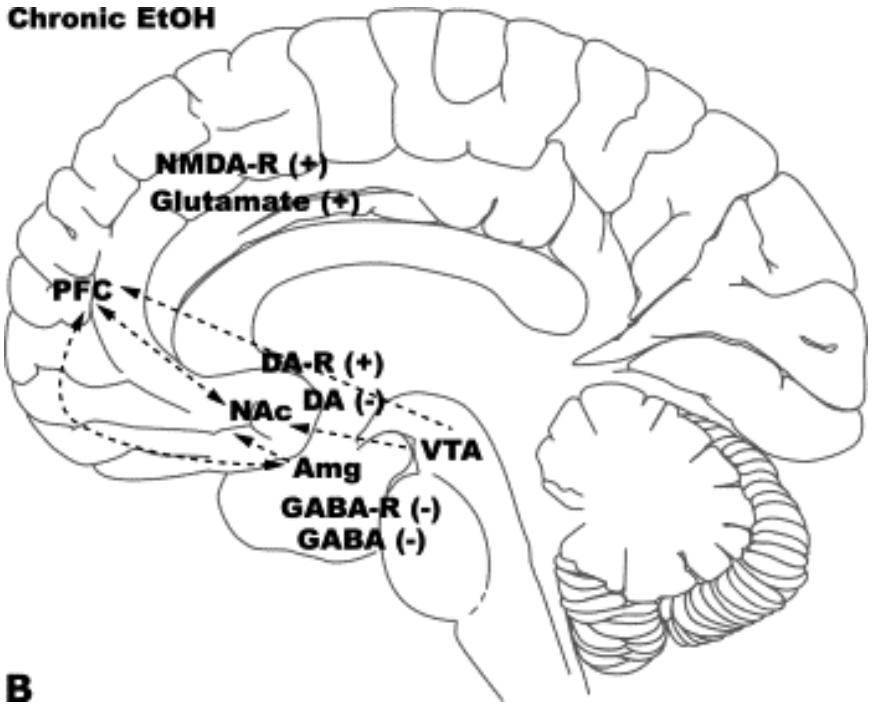


FIGURE 1.4 :THE MOLECULAR ADAPTATIONS IN THE MESOCORTICOLIMBIC PATHWAY OCCURRING WITH ALCOHOLISM

A. The mesocorticolimbic system includes several brain areas: prefrontal cortex (PFC), nucleus accumbens (NAc), ventral tegmental area (VTA), and amygdala (Amg). Alcohol consumption induces major effects on the dopaminergic, glutamatergic, and GABAergic systems in this pathway. Acute effects of alcohol include: increased dopamine release (associated with reward), increased GABA receptor activity (associated with anxiolysis, sedation, and motor incoordination), and decreased glutamate receptor activity. DA, dopamine; EtOH, ethanol; NMDA-R, N-methyl-D-aspartate receptor; GABA-R, g-aminobutyric acid receptor. B. Chronic alcohol consumption causes neuroadaptations to oppose the effects of acute alcohol and include: decreased dopamine release and increased dopamine receptor expression, decreased GABA receptor expression, increased glutamate release and increased NMDA receptor expression.

Dopamine

Dopamine is a neurotransmitter involved in reward related mechanisms in the brain. Dopamine is thought to contribute to alcoholism by signaling in the midbrain dopaminergic system, a brain circuit involved in associative learning, incentive salience, and reward prediction (Gonzales, Job et al. 2004). The midbrain dopaminergic system originates in the ventral tegmental area (VTA) and projects to regions of the brain such as

the striatum, nucleus accumbens (NAc), and prefrontal cortex (PFC). Alcohol and other drugs of abuse increase dopaminergic activity in the midbrain region of rodents and humans (Boileau, Assaad et al. 2003). Dopamine release in the midbrain partially mediates the positive-reinforcing properties of acute alcohol exposure necessary for the development of alcoholism (Raeder, Holter et al. 2008). Moreover, preference for alcohol has been directly correlated with alcohol-induced dopamine release in the midbrain. This is illustrated by the fact that rats which are bred to prefer alcohol release more dopamine than wild-type rats in an alcohol self-administration study (Weiss, Lorang et al. 1993). Also, mice lacking different dopamine receptors and transporters show modified alcohol preference compared with controls, further illustrating dopamine's involvement in alcohol-related behaviors (Crabbe, Phillips et al. 2006).

Acute alcohol exposure activates dopamine reward pathways, whereas chronic treatment produces a hypodopaminergic state associated with dysphoria, which can lead to craving and relapse (Koob and Volkow 2010). A 1-year-long chronic alcohol treatment in rats decreased dopamine and its metabolite in the striatum, decreasing tyrosine hydroxylase protein levels and increasing dopamine transporter protein levels compared to controls (Rothblat, Rubin et al. 2001). Positron emission tomography scans show that chronic alcoholics have fewer dopamine receptors of type D2 compared with non-alcoholics. D2 receptors in the striatum are mainly localized on GABA-synthesizing cells. These results provide evidence of GABAergic involvement in the dopaminergic abnormalities seen in alcoholics (Volkow, Wang et al. 2002). These results also reflect the brain's homeostatic mechanism to adapt to initial increases in dopamine levels after chronic exposure to alcohol.

The dopamine receptor antagonist fluphenazine will block alcohol self-administration when injected into the NAc (Rassnick, Pulvirenti et al. 1992). Additionally,

several clinical trials have shown that aripiprazole, an atypical antipsychotic, reduces craving and increases positive subjective feelings in alcoholics (Martinotti, Nicola et al. 2007, Brunetti, Di Tizio et al. 2012). Although these clinical trials are promising, the mechanism by which dopamine malfunction caused by alcohol contributes to clinical alcoholism is currently unknown.

GABA-A

GABA is the primary inhibitory neurotransmitter, and the GABA-A receptor, a ligand-gated chloride channel, is the most abundant inhibitory receptor in the mammalian brain. Acute alcohol exposure enhances GABA-A function. The molecular actions by which alcohol may exert its effects on GABAergic activity are by directly binding to the receptor, increasing presynaptic release of GABA or releasing GABAergic steroids (Lobo and Harris 2008). This acute action of alcohol on the GABAergic system, along with the effects of chronic alcohol use, is illustrated in Figure 1.5.

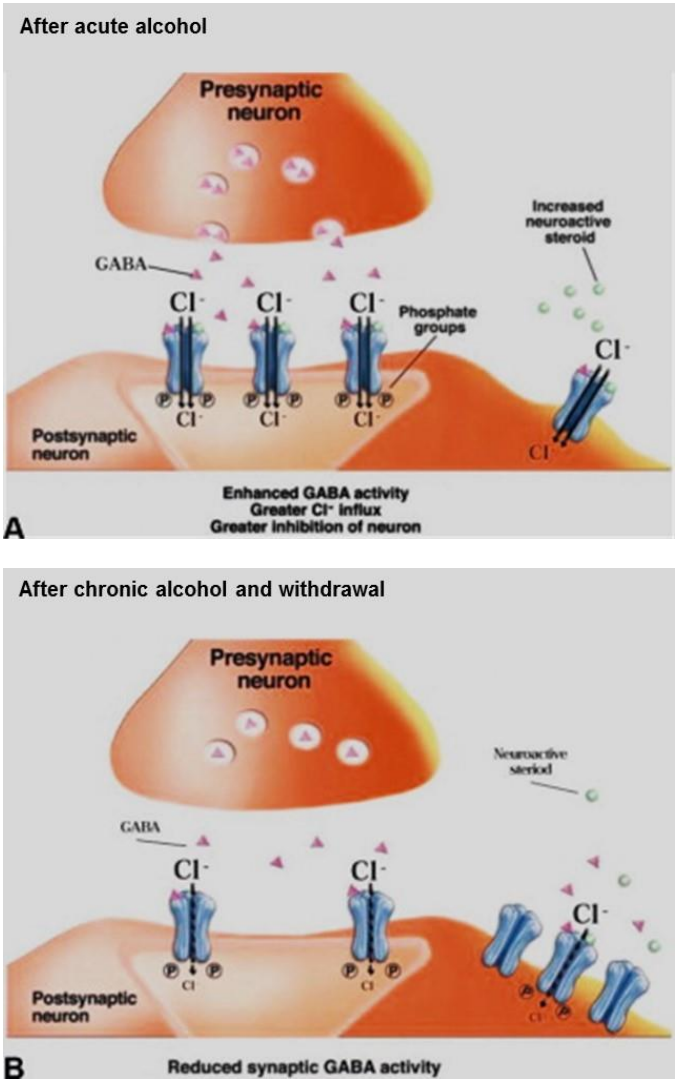


FIGURE 1.5: THE GABAERGIC SYSTEM AND THE EFFECTS OF ACUTE AND CHRONIC ALCOHOL EXPOSURE.

A. GABA acts in part through the activation of GABA-A receptors, which are ligand-gated ion channels that allow chloride ions (Cl-) to flow into the neuron after GABA binds to it. Greater influx of Cl- into the neuron makes it more difficult for the cell to generate a new action potential (which is why GABA is considered to be an inhibitory neurotransmitter). The molecular actions by which alcohol may exert its effects on GABAergic activity is by directly binding to the receptor, increasing presynaptic release of GABA, or releasing GABAergic steroids that activate extrasynaptic receptors . B. After chronic alcohol exposure and during withdrawal, GABA activity at the synapse is reduced, leading to reduced inhibition of the postsynaptic neuron. This results in development of anxiety and hyperexcitability. Figure has been adapted from Clapp et al., 2008.

Alcohol intoxication is characterized by anxiolysis, sedation, impaired cognitive function, hypnosis, and impaired motor function. Many of these behaviors are mimicked

by the administration of pharmacological GABA-A agonists like muscimol and benzodiazepines (Grobin, Matthews et al. 1998). Moreover, the effects of alcohol can be diminished by using pharmacological GABA antagonists like bucucilline and picrotoxin (Hyytiä and Koob 1995), suggesting that GABA-A signaling is directly involved in the acute actions of alcohol. Electrophysiological data support alcohol's actions on GABA-A receptors, showing potentiation of GABA-mediated chloride influx following alcohol administration in a variety of preparations. Mihic et al. 1997 identified a 45-amino-acid residue necessary and sufficient for the enhancement of GABA-A receptor function by alcohol, suggesting that alcohol's binding site is between the transmembrane 2 and the transmembrane 3 regions of the receptor (Mihic, Ye et al. 1997). This is supported by crystallographic analysis of alcohol binding to a related ligand-gated ion channel, GLIC (Sauguet, Howard et al. 2013). Chronic alcohol use causes changes in GABA receptor subunit composition in human alcoholics (Lewohl, Crane et al. 1997), and it should be noted that alcohol's pharmacological action on GABA-A receptors (and consequently behavioral responses) strongly depends on the subunit composition of the receptor. Alcohol increases GABAergic neurotransmission over a wide range of concentrations, with some studies showing that δ-containing receptors are more sensitive than other GABA-A receptors (Wallner, Hanchar et al. 2003). Use of mutant mice either lacking a subunit or containing an ethanol-resistant subunit demonstrated that the α2 and α3 subunits take part in mediating the motor-impairing effects of alcohol, and that the α2 subunit also takes part in the aversive properties of alcohol (Blednov, Borghese et al. 2011, Blednov, Benavidez et al. 2013).

Chronic alcohol use causes the downregulation of GABA-A receptors due to the initial overstimulation by the alcohol. In turn, glutamate receptors are upregulated to counteract the sedative effects of increased GABA-A. During withdrawal from alcohol, the

excess of glutamate receptors combined with the lack of GABA-A, causes anxiety, dysphoria, and in severe cases, seizures. Topiramate, an anticonvulsant seizure medication, was found to reduce voluntary alcohol intake in humans with alcohol dependence in recent clinical trials (Johnson, Rosenthal et al. 2008). Alcoholic patients achieved a rate of continuous abstinence much higher than those in a placebo group. They also reported fewer cravings compared to a placebo group but complained of other side-effects. Topiramate is associated with glutamate system inhibition, although the exact mechanism by which topiramate promotes abstinence is not yet understood.

GABA-B

GABA-B receptors are metabotropic receptors responsible for mediating slow inhibitory responses in the brain and have been implicated in alcoholism. Acute alcohol was seen to increase inhibitory GABA-B signaling in a concentration-dependent manner in rat midbrain dopaminergic neurons (Federici, Nistico et al. 2009). Silencing the GABA-B receptor in fruit flies using RNA interference decreases acute motor impairment after alcohol exposure (Dzitoyeva, Dimitrijevic et al. 2003). Similarly, in mice, cerebellar injections of GABA-B agonists accentuated and antagonists attenuated motor impairment following acute alcohol (Dar 1996). Conversely, recordings in CA1 pyramidal neurons have indicated that GABA-B signaling was immune to the acute effects of alcohol but became modulated after chronic exposure, perhaps because of neuroadaptations from dependence (Frye, Taylor et al. 1991). Baclofen, a GABA-B agonist prescribed to treat muscle spasticity, reduces relapse in dependent humans and decreases alcohol consumption in rats (Addolorato, Caputo et al. 2002, Maccioni and Colombo 2009, Agabio, Maccioni et al. 2012). Dr. Olivier Ameisen describes attending over 5000 AA meetings over the course of several years in an attempt to reduce his alcohol craving and abstain from drinking. Despite all efforts, he continued drinking and eventually treated

himself with high doses of baclofen; he was able to “cure” his disease (Ameisen 2005, Ameisen 2012). Preclinical studies show that baclofen is able to suppress withdrawal symptoms in alcoholics, promote alcohol abstinence, and reduce withdrawal-related anxiety and alcohol craving (Addolorato, Caputo et al. 2002). There is also evidence for baclofen having no significant effect on alcoholics (Garbutt, Kampov-Polevoy et al. 2010), suggesting that baclofen may be effective in treating only certain subtypes of alcoholics. There is still research to be done on baclofen and its side-effects, and therefore it has not yet been approved by the Food and Drug Administration (FDA) for the treatment of alcoholism (Leggio, Garbutt et al. 2010).

RNA splicing is a process that removes the intervening, non-coding sequences of genes (introns) from premRNA and joins the protein-coding sequences (exons) together in order to enable translation of mRNA into a protein. Sometimes, exons are extended or skipped, or introns are retained during the splicing process, leading to “alternative splicing.” Alternative splicing of the premRNA creates different splice variants for the same gene, which results in a functional diversity of proteins (Matlin, Clark et al. 2005). Eventually, the mRNA transcripts are exported to the cytoplasm and translated into different isoforms of the same protein that vary in their functional properties (Coetzee, Amarillo et al. 1999, Dredge, Polydorides et al. 2001). Drugs of abuse, such as alcohol, can affect alternative splicing. For example, chronic alcohol consumption enhances the complexity of GABA-B receptor splicing (Lee, Mayfield et al. 2010, Lee, Mayfield et al. 2014). Human alcoholics show differences in GABA-B receptor splice variants when compared to non-alcoholics, which may contribute to the pharmacological effects of GABA-B agonists such as baclofen in the treatment of alcoholism (Lee, Mayfield et al. 2014).

In summary, pharmacologic, behavioral, and electrophysiological evidence supports a role for GABA-B in mediating the effects of alcohol. Moreover, many of the behaviors associated with alcoholism result from the direct alcohol-induced modulation of GABAergic neurotransmission. It should be noted that the specific behaviors elicited by alcohol depend highly on receptor subtype expression and cellular location.

Serotonin (5-HT)

Serotonin is a neurotransmitter that serves many functions, including regulating mood, sleep, appetite, learning, memory, and other phenomena. The supply of neuronal serotonin originates mostly from neurons in the raphe nuclei whose axons innervate almost the entire brain, reflecting serotonin's vast physiological roles. There are many types of receptors for serotonin in the CNS and all are metabotropic, except for 5-HT3, which is a ligand gated ion channel. Evidence indicates that serotonin is involved in mediating both acute and chronic alcohol action and perhaps the development and maintenance of alcoholism.

Alcohol facilitates serotonergic transmission in part by increasing the potency for the agonist activation of the 5-HT3 receptor (Sung, Engel et al. 2000) and the open state duration of the channel (Zhou, Verdoorn et al. 1998). In general, an inverse relationship between serotonin transmission and alcohol drinking has been established. In animal models of drinking and in human alcoholics, increased serotonergic transmission is associated with less alcohol consumption while less serotonergic transmission is linked to more alcohol consumption (Lovinger 1997).

Electrophysiological recordings in neuroblastoma cells show that acute alcohol potentiates 5-HT3 receptor mediated ion currents (Lovinger and White 1991). Acute alcohol was also found to activate the 5-HT3 receptor in oocytes (Harris, Mihic et al.

1995), ganglion neurons (Lovinger and White 1991), frontal cortex neurons (Sung, Engel et al. 2000), and human embryonic kidney 293 cells (Lovinger and Zhou 1994).

Serotonin levels in animal brains are elevated after acute alcohol exposure (Murphy, McBride et al. 1982, LeMarquand, Pihl et al. 1994). Mice lacking the 5-HT1B serotonin receptor consume larger amounts of alcohol compared to wild-type. Mice lacking the receptor show much higher incoordination compared to wild-type mice (after just one injection of alcohol), suggesting this receptor is involved in the intoxication process (Crabbe, Phillips et al. 1996). The 5-HT2 receptor seems to be important for the reinforcing properties of alcohol because antagonists selectively decrease acute alcohol reinforcement (Roberts, McArthur et al. 1998). Blockade of the serotonin transporter (5-HTT) with fluoxetine, a selective serotonin reuptake inhibitor (SSRI) (a widely used antidepressant, also known as Prozac), or by genetic knockout, decreases alcohol consumption in rodents (Kelaï, Aïssi et al. 2003). Decreased consumption of alcohol following SSRI treatment has been observed in almost every rat model of alcoholism (Amit, Brown et al. 1977, Sellers, Higgins et al. 1992, Ciccocioppo, Economidou et al. 2006). Human studies have given less consistent results, but it appears that SSRIs may be effective in some subpopulations of alcoholics, such as late-onset individuals (Kranzler, Feinn et al. 2012).

In addition to enhancing 5-HT activity in the brain using SSRIs, another approach widely used to understand serotonin's effect on alcohol consumption has been to selectively block the 5-HT3 receptor. 5-HT3 antagonists decrease alcohol self-administration (Fadda, Garau et al. 1991, Hodge, Samson et al. 1993) and consumption in rodents (Sellers, Toneatto et al. 1994).

In humans, the drug ondansetron, a 5-HT3 antagonist that is FDA-approved for the treatment of chemotherapy-induced nausea, is a promising treatment for alcoholics. In a

clinical trial, alcoholics were randomly selected to receive either ondansetron or a placebo for 11 weeks. The ondansetron patients with early-onset alcoholism had fewer drinks per day and reported more days of total abstinence than the placebo group (Johnson, Roache et al. 2000). In another clinical trial with 71 alcoholic men receiving a 6-week treatment with a low dose of ondansetron (0.25 mg) showed significant levels of decreased drinking when compared to placebo (Sellers, Toneatto et al. 1994). In another study analyzing the reinforcing properties of alcohol and 5-HT3 receptor function, treatment with ondansetron decreased the subjective pleasurable effects of alcohol and the desire to drink (Johnson, Campling et al. 1993). Since the serotonin transporter is important for regulating the serotonergic system, alleles at the gene encoding 5-HTT might predict the severity of the alcoholism and the therapeutic response to treatment with ondansetron. A clinical trial with 283 alcoholics found that ondansetron recipients had fewer drinks per day and more days spent totally abstinent than those who received placebo. The effect was greater in alcoholics with the LL genotype than the SS or LS genotype of the 5'-regulatory region of the serotonin transporter gene (Johnson, Ait-Daoud et al. 2011). Moreover, animal studies have demonstrated that ondansetron might also be useful for treating opioid withdrawal symptoms (Pinelli, Trivulzio et al. 1997).

Big potassium (BK) channels

The BK channel is a high-conductance calcium- and voltage-dependent potassium channel (Atkinson, Robertson et al. 1991) that plays a dominant role in shaping neuronal activity and, unlike other voltage-gated ion channels, is strongly affected by alcohol (Treistman and Martin 2009). Tolerance, which is the loss of drug effectiveness over time, is an important component of addiction. Acute tolerance can have a rapid onset within minutes of alcohol exposure, whereas tolerance after prolonged alcohol exposure develops over the course of days or weeks. The degree of acute behavioral tolerance to

alcohol exhibited by a naïve subject can predict the likelihood of alcoholism (Treistman and Martin 2009). Thus, the determinants of acute tolerance are important to understand. The BK channel is a key target in the development of tolerance in invertebrates and mammals (Davies, Pierce-Shimomura et al. 2003, Martin, Hendrickson et al. 2008). There are several variables that influence the response of the BK channel to alcohol, including subunit composition, splice variant, and posttranslational mechanisms (covered in "The effects of alcohol on synaptic elements" below). BK channels from neurons of wild-type mice (in which the β4 subunit is well represented) exhibit little tolerance. By contrast, neuronal BK channels from β4 knockout mice do display acute tolerance. In addition to displaying tolerance, the β4 knockout mice drink more than their wild-type counterparts in an alcohol self-administration paradigm (Martin, Hendrickson et al. 2008).

Tolerance was found to involve the alternative splicing of the α subunit of the BK channel. The BK channel was found to have a sensitive and non-sensitive splice variant to alcohol. Within minutes of exposure to alcohol, the expression of the more alcohol-sensitive variant was selectively decreased, therefore producing tolerance (Treistman and Martin 2009). The development of acute tolerance to alcohol provides another example that demonstrates the effects of alternative splicing of mRNAs on alcohol-related behavior (seen previously with the GABA-B receptors).

Transcription factors

Transcription factors serve as a key mechanism by which distinct gene programs are controlled because they bind to highly specific DNA-regulatory sequences (control elements) (Renthal and Nestler 2008, Rahman 2012). Activation of alcohol-responsive transcription factors is also likely to result in changes in the expression of those genes with the corresponding control elements.

Heat shock factor protein 1 (HSF1) is a transcription factor which was found to be involved in the effects of acute alcohol application. Acute alcohol was found to increase GABA-A receptor α4 subunit mRNA by increasing the binding of HSF1 to the promoter regions of GABA, increasing its transcription. Blocking the expression of HSF1 reduced the alcohol-induced increases in the α4 subunit and conversely, an active form of HSF1 induced α4 subunit transcription in the absence of alcohol (Pignataro, Miller et al. 2007). Acute alcohol also facilitates activation of HSF1 to the promoter region of synaptotagmin-1, a protein involved in synaptic transmission and release, suggesting that alcohol has a direct control of neurotransmitter release in an acute fashion (Pignataro, Miller et al. 2007, Pignataro, Varodayan et al. 2009). HSF family of mRNA was found to be different between alcoholic and control postmortem frontal cortices (Lewohl, Wang et al. 2000), as well as in cultured cortical neurons exposed to chronic alcohol treatment (Wang, Krishnan et al. 2007). These results indicate that these transcription factors are also involved in alcohol dependence.

The activation of several signaling pathways involving cAMP, Ca^{2+} and extracellular signal regulated kinase lead to the phosphorylation of the transcription factor cyclic AMP response element-binding protein (CREB) (Winstanley, LaPlant et al. 2007). Some chromatin-remodeling enzymes target chromatin by interacting with specific transcription factors by guiding them to a specific locus on the DNA. When phosphorylated, the transcription factor CREB interacts with CBP, a HAT that helps facilitate target gene activation by acetylating neighboring histones. This process was found to play a major role in behavioral responses to cocaine (Levine, Guan et al. 2005, Malvaez, Mhillaj et al. 2011). Long-term treatment with alcohol was found to increase CREB transcription factor activation, which subsequently increases the binding of CREB to the promoter of the glutamate receptor, increasing the transcription of a specific

glutamate receptor subunit - NR2B. Site-directed mutation in the sequence where CREB binds, abolished the stimulatory effect by alcohol, suggesting that CREB is involved in mediating alcohol induced upregulation of the NR2B gene (Rani, Qiang et al. 2005). This is a great example of an alcohol-responsive transcription factor affecting downstream expression of receptors which are also involved in mediating the response to alcohol.

Another example suggesting that the downstream transcription of genes is selective to either the acute or chronic stages of addiction is that of the immediate-early genes, such as c-Fos and FosB. These immediate-early genes are induced rapidly in the brain by acute use of drugs such as cocaine and alcohol, whereas the transcription of genes such as Cyclin-dependent kinase 5 (Cdk5) and brain-derived neurotrophic factor (BDNF) is induced by chronic use of cocaine (Taylor, Lynch et al. 2007). Chronic use of drugs also causes the accumulation of ΔfosB, a member of the Fos family of transcription factors. ΔfosB accumulates in specific regions of the brain in response to drugs and persists for several weeks after the end of the stimulus (Watanabe, Henriksson et al. 2009, Damez-Werno, LaPlant et al. 2012). ΔfosB represents a molecular mechanism that can initiate and then sustain changes in gene expression that persist long after drug exposure ceases (Nestler, Barrot et al. 2001).

THE EFFECTS ON THE NEUROIMMUNE SYSTEM

Substantial evidence has accumulated that implicates an unlikely target, the neuroimmune system, in the development, progression, and persistence of alcoholism. The term neuroimmune refers to signaling molecules that were first associated with innate immunity but are also commonly found in the brain. The brain uses immune signaling systems as neuromodulators that have functions distinct from their role in the peripheral immune system and are critical for normal brain functions like neuronal

plasticity (Boulanger, Huh et al. 2001). One example of this is the major histocompatibility complex (MHC) class I molecule, which serves as a primary mediator of immune response in the periphery. However, it also modulates activity-dependent refinement and plasticity in cortical synapses and the developing visual system in the CNS (Glynn, Elmer et al. 2011, Elmer and McAllister 2012). Alcohol-related behavior can be affected by immune signaling that originates in the brain or immune signaling derived from the periphery that crosses the blood–brain barrier to act on the brain.

Ingesting alcohol activates the neuroimmune system (Crews, Zou et al. 2011), which is proposed to lead to further increases in alcohol consumption, producing an escalating feedforward loop not conducive to a homeostatic state. Alcohol consumption compromises the tight junctions in gut epithelium, allowing lipopolysaccharide (LPS), a Gram-negative bacterial endotoxin that is normally confined to the gut, to leak into the blood stream, where it binds to and activates Toll-like receptors (TLRs) expressed on liver Kupffer cells and other tissues. This initiates a signaling cascade that culminates in the release of proinflammatory cytokines in the blood stream that can then cross the blood–brain barrier and interact with the brain, thus affecting behavior. Researchers have found that alcohol increases TLR expression in the brain and increases its sensitivity to LPS (Crews, Qin et al. 2012, Vetreno and Crews 2012). To test how peripheral release of LPS would affect drinking behavior, Blednov et al. 2011 injected mice with LPS and found that a single injection produces long-lasting increases in alcohol consumption consistent with neuroimmune signaling, mediating the reinforcing properties of alcohol. The single injection of LPS also increased the firing rate of dopamine neurons in the ventral tegmental area, providing an example of how neuroimmune activation following peripheral LPS administration modulates brain reward circuitry (Blednov, Benavidez et al. 2011). Indeed, peripheral immune function has been found to be important for other

mental illnesses such as schizophrenia, depression, and autism (Dantzer, O'Connor et al. 2008, Kelley and Dantzer 2011, Derecki, Cronk et al. 2012, Jones and Thomsen 2013, Takao, Kobayashi et al. 2013); however, the mechanism by which peripheral immune activation exerts its effects on the brain is unknown and presents a field to be explored.

Immune signaling molecules are also found within the brain where they can be used for normal, non-immune signaling, but can also reflect pathology and lead to neurodegeneration if proinflammation is left unchecked (El Khoury 2010). Neurodegenerative effects of immune signaling have been demonstrated in Alzheimer's and Parkinson's disease (Carta and Pisanu 2013, Hickman and El Khoury 2013). Neurodegeneration has been observed in alcoholics and is especially prominent in the PFC (Fadda and Rossetti 1998). Neurodegeneration in the PFC can affect judgment and reasoning capabilities and further exacerbate chronic alcohol consumption. It is possible that neuroimmune activation from alcohol consumption could contribute to the neurodegeneration seen in alcoholics (Blanco, Valles et al. 2005, Hua, Ma et al. 2007, Alfonso-Loeches, Pascual-Lucas et al. 2010, Qin and Crews 2012).

Other evidence linking neuroimmune function with alcoholism is that immune gene expression in the brain was found to be altered in human alcoholics (Liu, Lewohl et al. 2006, Crews, Qin et al. 2012, Ponomarev, Wang et al. 2012), mice, and fruit flies after alcohol exposure (Qin, He et al. 2008, Kong, Allouche et al. 2010), and rodent genetic models of high alcohol consumption (Mulligan, Ponomarev et al. 2006, Saba, Bhave et al. 2006). Furthermore, mice lacking genes related to immune function show decreased alcohol consumption compared to littermate controls (Blednov, Ponomarev et al. 2012). Acute and chronic alcohol use activates microglia, the resident macrophages of the brain, and increases proinflammatory cytokines via nuclear factor κ B (NFκB) in the brain (Crews, Zou et al. 2011). Behavioral responses to acute alcohol were altered in mice that lack TLR

receptors, TLR2 or TLR4 (Wu, Lousberg et al. 2011). In a key publication, the knockdown of TLR4 in the rat amygdala decreased alcohol self-administration, demonstrating that neuroimmune signaling, independent of input from peripheral cytokines, was sufficient in regulating alcohol behavior (Liu, Yang et al. 2011).

Pioglitazone and rosiglitazone, agonists of the peroxisome proliferator activator receptor (PPAR) type γ, have anti-inflammatory properties largely mediated through their ability to inhibit the transcription factor NFκB and thus decrease proinflammatory cytokine production (Daynes and Jones 2002). Pioglitazone was found to reduce alcohol drinking, abolish reinstatement of alcohol seeking, reduce alcohol self-administration, and decrease the severity of physical withdrawal symptoms in rats (Stopponi, Somaini et al. 2011). Another PPAR agonist, clofibrate, prevents the acquisition of nicotine self-administration in naïve rats and monkeys and decreases nicotine self-administration in nicotine-dependent rats and monkeys, suggesting that the PPAR agonist might be viable options for treating the neuroimmune pathologies in alcoholism and other forms of addiction. The pharmacological blockade of neuroimmune activation reduces alcohol reward and decreases consumption using many types of anti-inflammatory drugs like minocycline, doxycycline, topiramate, anakinra, indomethacin, and CAPE (Agarwal 2001, Pascual, Blanco et al. 2007, Breslin, Johnson et al. 2010, Wu, Lousberg et al. 2011, McIver, Muccigrosso et al. 2012, Zalewska-Kaszubska, Bajer et al. 2013). Taken together, these findings have built substantial evidence for neuroimmune modulation of acute and chronic alcohol consumption and offer unique unexplored targets for therapeutic intervention.

Table 1.3 summarizes the neurotransmitter systems discussed in this chapter.

Neurotransmitter system	Associated reference
Glutamate	
Acute alcohol inhibits NMDA receptor function, while chronic use of alcohol upregulates NMDA receptor expression in the brain.	Lovinger et al., 1989 and Qiang and Ticku, 2005
Alcohol acts as a non-competitive inhibitor of the AMPA/Kainate receptors at high concentrations.	Dildy-Mayfield and Harris, 1992, Dildy-Mayfield and Harris, 1995 and Akinshola et al., 2003
MK-801, an NMDA antagonist, mimics the subjective effects of alcohol in animals.	Butelman et al., 1993
Ketamine, an NMDA antagonist, mimics the behavioral effects of alcohol in detoxified alcoholics.	Krystal et al., 1998
Chronic alcohol increases the expression of AMPA1 subunit (GluA1) in the brains of alcoholics.	Lewohl et al., 2000
NMDA receptors containing specific subunits are more sensitive to alcohol than others.	Raeder et al., 2008
Alterations in subunit composition of glutamate receptors were seen after chronic use of alcohol in mice.	Ortiz et al., 1995
Dopamine	
Acute alcohol exposure activates dopamine reward pathways, whereas chronic treatment produces a hypodopaminergic state associated with dysphoria, which could lead to craving and relapse.	Koob and Volkow, 2010
Alcohol increases dopaminergic activity in the midbrain region of rodents and humans.	Boileau et al., 2003
Dopamine release in the midbrain partially mediates the positive reinforcing properties of acute alcohol exposure.	Raeder et al., 2008
Alcohol-preferring rats release more dopamine than wild-type rats in response to alcohol self-administration.	Weiss et al., 1993
Mice lacking different dopamine receptors and transporters show modified alcohol preference compared with controls.	Crabbe et al., 2006
Chronic alcohol treatment decreases dopamine and its metabolite in the striatum, decreases tyrosine hydroxylase protein levels, and increases dopamine transporter protein levels in rats compared to controls.	Rothblat et al., 2001
PET scans show that chronic alcoholics have fewer D2 receptors when compared with non-alcoholics.	Volkow et al., 2002
The dopamine receptor antagonist fluphenazine will block alcohol self-administration when injected into the nucleus accumbens.	Rassnick et al., 1992
Aripiprazole, an atypical antipsychotic, reduces craving and increases positive subjective feelings in alcoholics.	Martinotti et al., 2007 and Brunetti et al., 2012
GABA-A	
Behavioral intoxication is mimicked by the administration of pharmacologic GABA-A agonists like muscimol and benzodiazepines.	Grobin et al., 1998
The effects of alcohol can be diminished by using pharmacologic GABA antagonists like bicuculline and picrotoxin.	Hyytia and Koob, 1995
Chronic alcohol use causes changes in GABA receptor subunit composition in human alcoholics.	Lewohl et al., 1997
Alcohol increases GABAergic neurotransmission over a wide range of concentrations, with some studies showing that delta-containing receptors are more sensitive than other GABA-A receptors.	Wallner et al., 2003
α2 and α3 subunits take part in mediating the motor-impairing effects of alcohol.	Blednov et al., 2011b
α2 subunit takes part in the aversive properties of alcohol.	Blednov et al., 2013

GABA-B	
Acute alcohol increases inhibitory GABA-B signaling in a concentration-dependent manner in rat midbrain dopaminergic neurons.	Federici et al., 2009
Silencing the GABA-B receptor in fruit flies decreases acute motor impairment after alcohol exposure.	Dzitoyeva et al., 2003
Cerebellar injections in mice of GABA-B agonists accentuated and antagonists attenuated motor impairment following acute alcohol.	Dar, 1996
CA1 pyramidal neuron recordings indicated that GABA-B signaling was immune to the acute effects of alcohol but became modulated after chronic exposure.	Frye et al., 1991
Baclofen, a GABA-B agonist, reduces relapse in dependent humans and decreases alcohol consumption in rats.	Addolorato et al., 2002a, Addolorato et al., 2002b, Maccioni and Colombo, 2009 and Agabio et al., 2012
GABA-B has complex alternative splicing leading to several splice variants in the alcoholic brain.	Lee et al., 2013
Serotonin (5-HT)	
Acute alcohol potentiates 5-HT3 receptor.	Lovinger, 1991, Lovinger and White, 1991 and Lovinger and Zhou, 1994; Harris et al., 1995 and Sung et al., 2000
Serotonin levels in animal brains are elevated after acute alcohol exposure.	Murphy et al., 1982 and LeMarquand et al., 1994
Mice lacking the 5-HT1B serotonin receptor consume larger amounts of alcohol compared to wild type.	Crabbe et al., 1996
5-HT2 antagonists selectively decrease acute alcohol reinforcement.	Roberts et al., 1998
Blockade of the serotonin transporter (5-HTT) with selective serotonin reuptake inhibitors (SSRIs), or by genetic knockout, decreases alcohol consumption in rats and partially in humans.	Brown et al., 1979, Sellers et al., 1992, Kelai et al., 2003, Ciccocioppo et al., 2006 and Kranzler et al., 2012
Selective antagonists of the 5-HT3 receptor decrease alcohol self-administration and consumption in rodents.	Fadda et al., 1991, Hodge et al., 1993 and Sellers et al., 1994
Selective antagonists of the 5-HT3 receptor decrease the amount of drinks per day and increase the amount of abstinence time in alcoholics. Also, decreases the subjective pleasurable effects of alcohol and the desire to drink.	Johnson et al., 1993, Johnson et al., 2000 and Sellers et al., 1994

TABLE 1.3: SUMMARY OF NEUROTRANSMITTER SYSTEMS DISCUSSED IN THE FIRST SECTION OF THIS CHAPTER

DA (dopamine); PET (positron emission tomography).

The Effects of Alcohol on Synaptic Elements

Chronic alcohol use causes changes in synaptic structure and function which is believed to be caused by persistent changes of many genes. Local translation of mRNAs in the synapse may be responsible for the neuroadaptations resulting from chronic alcohol use and abuse. Over the past decade, several microRNAs have been proposed as

playing a key role in regulating local mRNA translation. As 'master regulators' of many mRNAs, changes in microRNAs could account for the systemic alterations in mRNA and protein expression observed in drug abuse and dependence. There is limited evidence, however, regarding how synaptic microRNAs control local mRNA translation during chronic drug exposure and how this contributes to the development of dependence.

Because alcohol dependence is tightly linked to changes in the expression of many genes in the brain, an effective therapy must therefore rely on the ability to target multiple alcohol-related genes. Recent studies indicate that manipulation of microRNAs affects addiction-related behaviors such as the rewarding properties of cocaine, cocaine-seeking behavior, alcohol consumption and preference over water. However, little is known about the synaptic microRNAs involved in the regulation of mRNA translation in alcohol dependence. Here, I present the current knowledge regarding the microRNA composition in the synapse, the regulatory actions over synaptic mRNA, and the resulting effect on chronic alcohol consumption in mice.

Local Translation and mRNAs

Chronic drug abuse induces long-term changes in brain gene and protein expression, which likely contribute to the neuropathologies associated with abuse and dependence (Nestler 2001, Kauer and Malenka 2007). Drug-induced transcriptional reprogramming in the brain may account for some of the effects of repeated drug exposure (Mayfield, Harris et al. 2008, Robison and Nestler 2011, Ron and Messing 2013). Ultimately, neuroadaptations due to chronic drug use are controlled by the regulation of many genes expressed within individual neurons or glial cells (Farris and Miles 2012). At the cellular level, changes in molecular pathways originate from changes in gene expression and translation of proteins. Re-organization of synaptic structure and function

is one manifestation of these changes. Many of the functional pathways that are altered in addiction paradigms include growth factors (Ron and Messing 2013), serine-threonine kinases (Sanna, Simpson et al. 2002, Lesscher, Wallace et al. 2009), glutathione pathway enzymes, protein translation (Neasta, Ben Hamida et al. 2010, Barak, Liu et al. 2013), and inflammatory pathways (Blednov, Benavidez et al. 2011, Gorini, Harris et al. 2014).

Proteins can be directly translated in synaptic regions, allowing cells to rapidly respond to stimuli and bypassing the need to transport proteins to the synapse, an energetically costly and slow process. Translation is an important mechanism underlying synaptic plasticity and is controlled locally in response to environmental signals (Ule and Darnell 2006, Schratt 2009). Synaptic translation is one way by which drugs of abuse induce targeted neuroadaptations (Nunez and Mayfield 2012). Drug-induced neuroadaptations and transcriptional changes influence a complex regulatory network that controls how and when the synaptic mRNAs are translated (Smalheiser and Lugli 2009). MicroRNAs, in part, regulate local protein synthesis and the molecules that control it. Many of the characteristic alterations in synaptic composition due to chronic drug exposure may arise from alterations in microRNAs (Eipper-Mains, Kiraly et al. 2011). This section of the thesis examines how microRNAs regulate synaptic translation and how this relates to the molecular pathways in drug use disorders.

Synaptic translation occurs in response to neural activity following chemical changes in the extracellular milieu. The first evidence came from electron micrographs showing clusters of polyribosomes at the synapse (Steward and Levy 1982). Twenty years later, the first dynamic visualization of localized protein synthesis was demonstrated when Aakalu et al. 2001 definitively showed translation within isolated dendrites in response to BDNF (Aakalu, Smith et al. 2001). In addition, application of dopamine to cells induced local protein translation (Smith, Starck et al. 2005). Discrete increases in protein

levels can occur in as little as 5 minutes, as shown for activity-regulated cytoskeleton-associated protein (Arc) (Niere, Wilkerson et al. 2012), or in 20 minutes for Ca2+/calmodulin-dependent protein kinase II (CaMKII)(Gong, Park et al. 2006).

Location- and time-specific translation at an activated synapse allows for spatially restricted expression of new proteins (Wang, Martin et al. 2010). This specialized control works to fine-tune translational mechanisms (Jung, Gkogkas et al. 2014). Newly translated proteins have fewer post-translational modifications, and thus will have different signaling properties than older proteins (Jung, Gkogkas et al. 2014). All of these factors help ensure the rapid and targeted responses required for neuronal signaling, and perturbations to this balanced system can profoundly alter cellular pathways.

Some of the first evidence of synaptic translation came from synaptoneurosome (SN) preparations that enrich for pre-and post-synaptic compartments of neurons, astrocytes, oligodendrocytes, and microglia (Hollingsworth, McNeal et al. 1985). This preparation can be used to study the synaptic transcriptome (Hollingsworth, McNeal et al. 1985, Quinlan, Philpot et al. 1999) and synaptic translation of mRNAs (Raab-Graham, Haddick et al. 2006, Sosanya, Huang et al. 2013). The development of this preparation, together with advances in microscopy and molecular cloning, have facilitated the discovery of the regulatory processes that govern mRNA translation at the synapse.

SNs and post-synaptic enriched preparations helped identify the method by which mRNA is distributed into synaptic compartments. The targeting of mRNA to dendrites occurs through the 3'UTR (Kislauskis and Singer 1992, Mayford, Baranes et al. 1996, Aakalu, Smith et al. 2001, Miller, Yasuda et al. 2002, Martin and Ephrussi 2009). The 3'UTR contains targeting elements that direct where and how mRNA is translated. Evidence suggests that the targeting of mRNA to the dendrite relies on cis-acting elements, often called zip codes (Meer, Wang et al. 2012). Cis-acting elements are recognized by trans-

acting factors for proper dendritic targeting and regulation. For example, localization of the β-actin protein requires a 54-nucleotide cis-acting zip code in the 3'UTR to target it to dendrites and growth cones (Kislauskis and Singer 1992, Zhang, Singer et al. 1999, Eom, Antar et al. 2003).

The zip codes can be single- or double-stranded stem loop structures comprised of multiple independent cis-acting elements that aggregate and confer distinct localization properties for each mRNA (Jambhekar and Derisi 2007, Holt and Bullock 2009). Cis-acting elements are normally within the 3'UTR but have also been found in the coding region and the 5'UTR. Regulation of local translation occurs through extensive interactions between the 3'UTR zip codes of mRNAs and microRNAs in concert with RNA-binding proteins. Drug-induced alterations in microRNAs may affect mRNA translation and distribution in the synapse via these mechanisms.

Local Translation and microRNA Regulation

MicroRNAs comprise a specific class of small non-coding RNAs that bind to complementary sequences on target mRNAs to repress translation and silence gene expression (Ambros 2001, Lee and Ambros 2001). MicroRNAs can regulate translation of many genes at once, making them 'master regulators' of cellular gene expression. They are highly abundant in the brain and play important roles in multiple biological processes, including brain development (Krichevsky, King et al. 2003), synapse formation (Schratt, Tuebing et al. 2006), synaptic plasticity (Smalheiser and Lugli 2009, Cohen, Lee et al. 2011), neuroimmune signaling (Soreq and Wolf 2011), learning and memory (Gao, Wang et al. 2010, Konopka, Kiryk et al. 2010) and mental disorders (Beveridge, Tooney et al. 2008, Parsi, Smith et al. 2015).

MicroRNAs control both translational repression and degradation, and they also act in concert with RNA-binding proteins to pinpoint their target mRNAs which often occurs through interaction with cis-acting elements. MicroRNAs are transcribed in the nucleus as pri-microRNA. They are then cleaved by Drosha into pre-microRNA and then transported into the cytoplasm. Alternatively, they may be spliced from introns in other genes, and then folded into pre-microRNA. Dicer then completes processing in the cytoplasm and assembly into the RNA-induced silencing complex (RISC) as dsRNA (Iwasaki, Kobayashi et al. 2010). The RISC complex retains the strand of microRNA with the lowest free energy at the 5'UTR which can bind to its target mRNAs. MicroRNAs associate with Argonaute (Ago) in the RISC complex to target their mRNAs (Bartel 2009). MicroRNAs need only contiguous pairing of the 'seed' region (nucleotides 2–7) to successfully pair with an mRNA (Stark, Brennecke et al. 2005, Nielsen, Shomron et al. 2007). However, different binding patterns have been observed that may alter the target affinity of the microRNA. Because of this, microRNAs can target and bind multiple mRNAs, and mRNAs can have multiple microRNA regulatory sites (Goldie and Cairns 2012).

MicroRNAs repress translation by blocking ribosomal interaction with target mRNA, by preventing interaction of Eukaryotic initiation factor (eIF4E) with mRNA, or by targeting mRNAs to P-bodies (processing bodies) for degradation (Pillai, Bhattacharyya et al. 2005, Mathonnet, Fabian et al. 2007, Filipowicz, Bhattacharyya et al. 2008). Degradation occurs when mRNAs de-circularize and ribosomes dissociate. MRNA-microRNA interactions are reversible, allowing activity-dependent conditions to dictate which mRNAs are targeted by the RISC (Filipowicz, Bhattacharyya et al. 2008).

MicroRNAs target the cis-acting elements in the 3'UTRs of mRNAs, similar to how RNA-binding proteins operate. Several systems portray a 'push–pull' mechanism of inhibiting and/or promoting translation in which both microRNAs and RNA-binding

proteins participate. Kv1.1, a voltage gated potassium channel, is regulated by both HuD, an RNA-binding protein, and by miR-129, and this occurs in response to mTOR activation (Sosanya, Huang et al. 2013). Kv1.1 is translated in dendrites only when mTOR activity is low (Raab-Graham, Haddick et al. 2006). Blocking mTOR activity releases Kv1.1 from miR-129 repression and frees HuD from higher affinity targets, enabling HuD to initiate translation of Kv1.1. Moreover, NMDAR activity alters the expression levels of multiple microRNAs, including those that inhibit mTOR inhibitors (Kye, Neveu et al. 2011). In addition, miR-125 is bi-directionally regulated by mGluR activity (Muddashetty, Nalavadi et al. 2011). MiR-125a regulates the expression of post synaptic density protein (PSD-95) in response to mGluR, and the process involves the formation of an inhibitory complex between miR-125a and Ago2 (Muddashetty, Nalavadi et al. 2011). These examples demonstrate that local translational control by microRNAs is dependent on the activity conditions and the coordinated work of other proteins.

MicroRNA translational regulation may play a prominent role in diseases such as temporal lobe epilepsy where neuronal activity is high. Silencing of miR-134 in a rat model of epilepsy decreased the number of spontaneous seizures. The seizure-suppressive effects implicate a neuroprotective role for some microRNAs in the brain (Jimenez-Mateos, Engel et al. 2012). In Parkinson's disease, disruptions of microRNA processing involving dicer produce an upregulation of mRNAs in dendrites of dopaminergic neurons, and symptoms can be alleviated by reintroducing functional microRNAs (Gibbings, Leblanc et al. 2012, Gibbings, Mostowy et al. 2013, Heman-Ackah, Hallegger et al. 2013). Conversely, increasing levels of miR-125b induces cognitive defects in mouse models of Alzheimer's disease (Banzhaf-Strathmann, Benito et al. 2014). These studies suggest that microRNAs provide a crucial link between cellular activity and rapid, reversible control of mRNAs in disease states (Bhattacharyya, Habermacher et al. 2006). The remainder of this

thesis section will discuss the role of microRNAs and local mRNA targets in response to different drugs of abuse.

MicroRNAs Regulation Following Exposure to Stimulants

Stimulants are psychoactive substances that increase the activity of the nervous system. Cocaine and amphetamines interact directly with the dopamine transporter, blocking dopamine reuptake into presynaptic terminals, thus increasing the dopamine levels in the synapse. Dopamine exerts positive effects on the local synthesis of glutamate receptors, possibly enhancing drug-induced reward by stimulating the VTA. Dopamine D1/D5 receptor activation stimulates protein synthesis in dendrites of cultured hippocampal neurons and increases GluA1 synaptic expression (Smith, Starck et al. 2005). Miniature excitatory post-synaptic currents (mEPSCs) increased in frequency within 15 minutes after application of a dopamine agonist which correlated with the increased GluA1. In a second study, this lab examined the effects of blocking the glutamatergic transmission of action potentials locally by blocking NMDAR in dendrites while action potentials were blocked globally with the sodium channel blocker tetrodotoxin (Sutton, Wall et al. 2004). The loss of NMDAR signaling in dendrites increased the expression of calcium permeable AMPAR at synapses, resulting in a rapid increase in mEPSC amplitude, complementary to the increase in synaptic AMPARs. The results suggest a homeostatic role for tonic NMDAR activity that actively controls some types of protein synthesis and suggest that the sensitivity of the dendritic glutamatergic system is due in large part to rapid, local changes in protein synthesis.

Within the VTA, cocaine induces immediate changes in synapse composition and increases excitability through an increased number of AMPARs (Bellone and Luscher 2006). Activation of mGluRs leads to long-term depression (LTD) at many brain synapses

(Malenka and Bear 2004), and mGluR-LTD in the VTA efficiently reverses cocaine-induced strengthening of excitatory inputs onto dopamine neurons (Bellone and Luscher 2006). It was later demonstrated that the mGluR-LTD mediated reversals in cocaine-induced excitability occur through an exchange of GluA2-lacking AMPARs for GluA2-containing receptors (Mameli, Balland et al. 2007). Synaptic insertion of GluA2 depends on rapid protein synthesis of GluA2 mRNA through the mTOR pathway (Mameli, Balland et al. 2007), the pathway discussed above in alcohol-related memories. Overall, the dynamic expression of glutamate receptors at post-synaptic synapses is important for neuroadaptations following drugs of abuse (Saba, Storchel et al. 2012), and the glutamate system also plays a key role in protein synthesis-dependent forms of synaptic plasticity.

Cocaine regulates protein synthesis in multiple brain regions. For example, cocaine elevates levels of BDNF in the NAc, a key region in the reward circuit. Grimm et al. 2003 showed a time-dependent increase in BDNF expression as well as increases in cocaine craving in response to a protracted abstinence period (Grimm, Lu et al. 2003). BDNF inhibition in the NAc decreases cocaine-seeking (Graham, Edwards et al. 2007). In contrast, BDNF injections into the medial PFC (mPFC) decreases cocaine self-administration (Berglind, Whitfield et al. 2009), drug seeking (Sadri-Vakili, Kumaresan et al. 2010), and cue- and priming-induced reinstatement (Berglind, Whitfield et al. 2009). BDNF injection into the NAc core, but not the shell, causes protein synthesis- and kinase-dependent increases in cell surface GluA1 30 minutes post-injection. GluA2 and GluA3 were unaffected, suggesting an effect of BDNF on homomeric GluA1 calcium permeable AMPARs (Li and Wolf 2011). BDNF injections into the VTA and NAc also produce persistent enhancement of cocaine-seeking (Lu, Dempsey et al. 2004). These results demonstrate that exogenous BDNF rapidly increases AMPAR surface expression in the rat NAc core, supporting an interaction between increases in endogenous BDNF levels and alterations

in AMPAR transmission in cocaine-experienced rats (Li and Wolf 2011). As discussed earlier, alcohol also affects BDNF expression levels and BDNF may regulate alcohol consumption and reward.

CREB is a candidate for mediating some of the neuroadaptations following drugs of abuse. The role of CREB in the rewarding properties of cocaine and methamphetamines was investigated using CPP to measure reward memories (Kuo, Liang et al. 2007). The drugs were injected in one of three compartments of the animal cage, and the time spent in the drug-injected compartment was compared to the saline-injected and drug-free compartments. If the drug is rewarding, the animal will choose to spend longer periods of time in the drug-injected compartment. Cocaine-induced CPP (2.5–5.0 mg/kg/dose) was abolished by pretreatment with a protein synthesis inhibitor, whereas methamphetamine-induced (0.5 or 1.0 mg/kg/dose) CPP was not affected by the pretreatment. Moreover, post-treatment with a protein synthesis inhibitor (2 h after each drug-place pairing) disrupted cocaine- but not methamphetamine-induced CPP. Increased CREB levels in NAc were associated with cocaine, but not methamphetamine, rewarding memories. Intra-NAc CREB antisense infusion diminished cocaine- but not methamphetamine-induced CPP. Taken together, the data show cocaine- but not methamphetamine-associated memory formation requires de novo protein synthesis.

The studies above highlight the various pathways through which drugs of abuse modulate local protein synthesis. Alteration in the glutamatergic system is an example of the dynamic changes in synaptic receptor composition and function following drugs of abuse. The intricate ways in which BDNF alters the effects of alcohol and cocaine suggest that it has region-specific roles which rely upon its ability to alter synaptic composition by interacting with local protein synthesis. Furthermore, BDNF is a downstream target of

various drugs in different brain regions and may represent a common target for treating drug dependence.

Stimulants such as cocaine and amphetamines increase the levels of dopamine in the synapse in both humans and animals (Di Chiara and Imperato 1988). MiR-181a expression is induced by exposure to dopamine, cocaine, and amphetamines in NAc (Saba, Storchel et al. 2012). MiR-181a was enriched in synapses following cocaine administration (Chandrasekar and Dreyer 2009). Using bioinformatics tools, Chandrasekar and Dreyer (2009) detected conserved binding sites for miR-181a within the mRNA encoding for the GluA2 subunit of AMPARs and subsequently showed that both overexpression and knockdown of miR-181a regulates GluA2 translation (Figure 1.6). Decreased GluA2 expression coincided with decreased spine formation and mEPSCs. MiR-181a overexpression increased cocaine-induced CPP, while knockdown of miR-181a produced the opposite effect (Chandrasekar and Dreyer 2011). Taken together, these results identify miR-181a as a key synaptic regulator of mammalian AMPARs with the potential to regulate drug-induced synaptic plasticity (Jonkman and Kenny 2013).

Over-expression of let-7, a microRNA that is decreased in response to cocaine, attenuated cocaine-induced CPP (Chandrasekar and Dreyer 2011). Let-7 targets CREB and BDNF, and cocaine-induced decreases in let-7 increases the expression of its targets (Chandrasekar and Dreyer 2011).

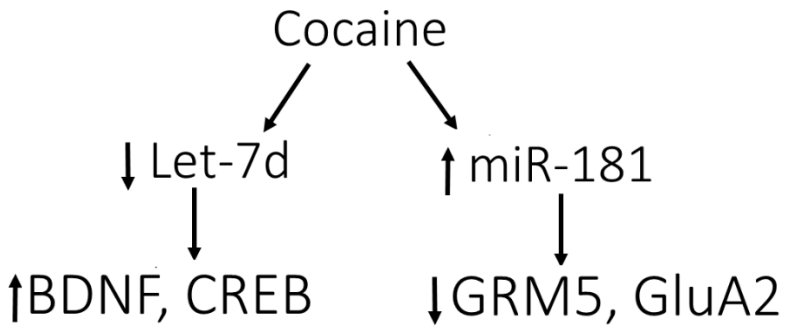


FIGURE 1.6: MODEL FOR COCAINE-INDUCED MICRORNA-MRNA INTERACTIONS

Cocaine causes the downregulation of let-7d, resulting in induction of its target genes, BDNF and CREB. In contrast, miR-181a is upregulated by cocaine. Figure and legend were modified from the original in Jonkman and Kenny (2013).

The X-linked transcriptional repressor, methyl CpG binding protein 2 (MeCP2), plays an important role in Rett syndrome, a form of mental retardation. MeCP2 translation has been found to be regulated by miR-132. Blocking miR-132 activity increased MeCP2 and BDNF levels in cultured rat neurons, and the loss of MeCP2 reduced BDNF and miR-132 levels in vivo (Klein, Lioy et al. 2007). Further studies showed that MeCP2 facilitates cocaine intake in rats with extended access to the drug, and this depends on interactions with miR-212, a family member of miR-132. The relationship between MeCP2 and miR-212 mediates the cocaine-induced effects on BDNF levels (Figure 1.7) (Im, Hollander et al. 2010). Moreover, miR-212 decreases responsiveness to the motivational properties of cocaine (Hollander, Im et al. 2010). These findings suggest a mechanism by which microRNA homeostatic control of MeCP2 and BDNF expression affects cocaine intake and related behaviors. The role of microRNAs (and mRNAs) in regulating the BDNF system is important in both cocaine and alcohol action.

MiR-124 and let-7d are significantly downregulated in the striatum after chronic cocaine administration. Decreased BDNF and dopamine D3 receptor mRNA and protein levels were regulated by miR-124 and let-7d, respectively (Chandrasekar and Dreyer

2009). Overall, brain-specific microRNA-mRNA interactions are altered by drugs of abuse, and if identified, could be used as important tools in future therapeutic studies.

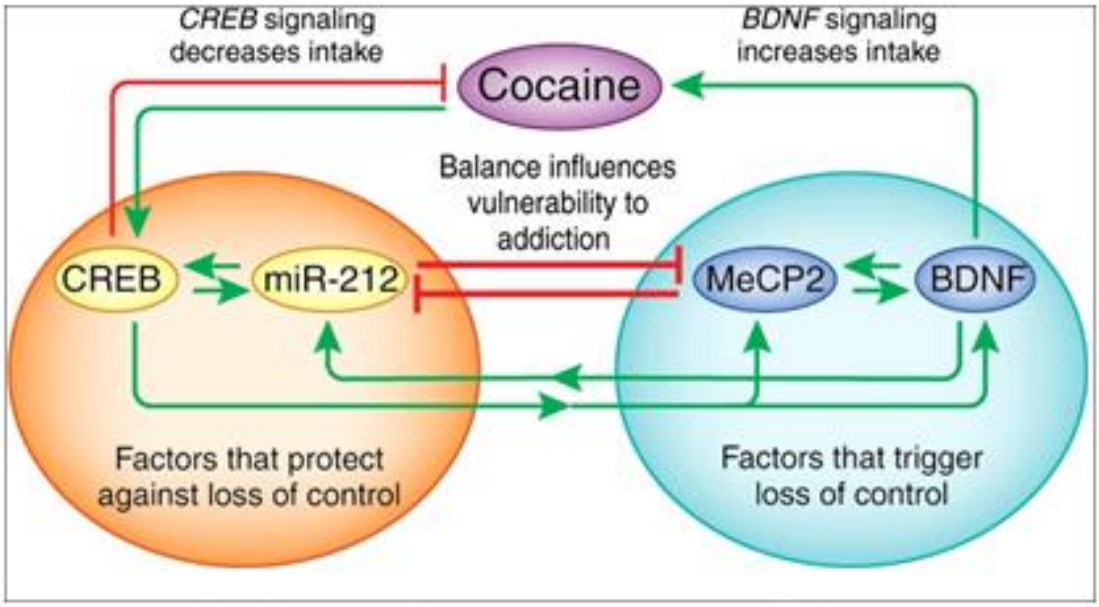


FIGURE 1.7: THE INTERACTIONS BETWEEN MIR-212, CREB, METHYL CPG-BINDING PROTEIN 2 (MECP2), AND BDNF

Cocaine activates CREB-miR-212 and MeCP2-BDNF signaling and the balance between these pathways likely regulates escalation of cocaine intake ('loss of control'). Figure has been adapted from Jonkman and Kenny (2013).

MicroRNA Regulation Following Alcohol Exposure

Overall, many microRNAs are upregulated in the human alcoholic brain (Lewohl, Nunez et al. 2011). Some of these alcohol-responsive microRNAs overlap with known local translational pathways.

As discussed earlier, the BK channel is a well-established alcohol target (Dopico, Anantharam et al. 1998) and an important contributor to behavioral and molecular alcohol tolerance (Davies, Pierce-Shimomura et al. 2003). Alcohol was found to

upregulate miR-9, which in turn regulates the expression of the BK channel subunits. This leads to post-transcriptional reorganization of BK splice variants and results in downregulation of the specific splice variant that is sensitive to alcohol. This mechanism is proposed to mediate development of cellular tolerance to alcohol (Pietrzykowski, Friesen et al. 2008).

A persistent upregulation of miR-206 expression was observed in mPFC, but not VTA, amygdala or NAc after 3 weeks of withdrawal from a 7-week exposure to alcohol vapor (Tapocik, Solomon et al. 2013). Overexpression of miR-206 in the mPFC of non-dependent rats reproduced the escalation of alcohol self-administration seen following a history of dependence and significantly inhibited BDNF expression (Tapocik, Barbier et al. 2014). BDNF expression was repressed by miR-206, but not miR-9, in a 3'UTR reporter assay, confirming BDNF as a functional target of miR-206. Furthermore, the decreased expression was dependent on the presence of all three miR-206 target sites in the 3'UTR of BDNF (Tapocik, Barbier et al. 2014). These results implicate miR-206 and BDNF in escalation of alcohol consumption which is a hallmark of alcoholism. Thus, both microRNAs and mRNAs (discussed earlier) regulate alcohol consumption via BDNF signaling.

Some microRNAs are downregulated by alcohol exposure, such as miR-382 in NAc (Li, Li et al. 2013). MiR-382 directly targets the dopamine receptor D1 (DRD1) and can modulate the expression of ΔfosB. Overexpression of miR-382 attenuated the alcohol induced upregulation of DRD1 and ΔfosB, decreased voluntary alcohol intake and preference and inhibited the DRD1-induced action potentials.

Bahi et al. 2013 showed that miR-124a was downregulated in the dorso-lateral striatum (DLS) following alcohol drinking. Silencing miR-124a attenuated ethanol-induced CPP as well as voluntary alcohol consumption. Moreover, overexpression of miR-124a

enhanced ethanol-induced CPP as well as voluntary alcohol consumption in a two-bottle choice drinking paradigm. Importantly, none of these treatments had an effect on saccharin and quinine intake (Bahi and Dreyer 2013).

Some microRNAs are upregulated in response to alcohol (Lewohl, Nunez et al. 2011). Darcq et al. 2015 showed that a mouse paradigm for binge alcohol drinking caused an upregulation of miR-30a-5p in the mPFC of mice. Overexpression of miR-30a-5p produced an escalation of alcohol intake and a preference over water. Conversely, inhibition of miR-30a-5p decreased excessive alcohol intake (Darcq, Warnault et al. 2014).

A single microRNA has the potential to target many genes (Nunez and Mayfield 2012, Tapocik, Solomon et al. 2013), and multiple microRNAs can cooperate to target the same genes (Grimson, Farh et al. 2007, Lewohl, Nunez et al. 2011). This mechanism may be of particular importance in the synaptic proteome where slight adaptations can greatly impact synaptic plasticity. For example, miR-7 and miR-153 were found to be differentially expressed between human alcoholics and controls. Interestingly, miR-7 and miR-153 were found to both regulate the expression of a-synuclein (Doxakis 2010). a-synuclein is a protein that plays a major role in neurotransmitter release in presynaptic terminals (Liu, Ninan et al. 2004, Greten-Harrison, Polydoro et al. 2010) and is involved in dopaminergic neurotransmission and neurodegenerative disorders (Doxakis 2010). Studies show that alcohol dependence in humans, as well as in rodents, is related to levels of a-synuclein (Bonsch, Greifenberg et al. 2005, Bonsch, Lenz et al. 2005). Interestingly, overexpression of miR-7 and miR-153 significantly reduces endogenous α-synuclein levels, whereas inhibition of miR-7 and miR-153 enhances translation of α-synuclein. These findings illustrate a mechanism by which alcohol changes the expression of two microRNAs and how they can cooperate to target an mRNA that is known to be involved in alcoholism as

well. This cooperation between the microRNAs allows regulation of gene expression with a reduced number of active microRNAs.

Summary

Excessive alcohol consumption causes widespread persistent changes throughout the brain. These molecular and cellular adaptations are thought to be the mechanisms by which neurons adapt to chronic alcohol use. These changes can eventually lead to alcoholism, depending on several factors, such as genetic predisposition, sex and environmental factors such as stress, age of drinking onset, and access to alcohol (Figure 1.8).

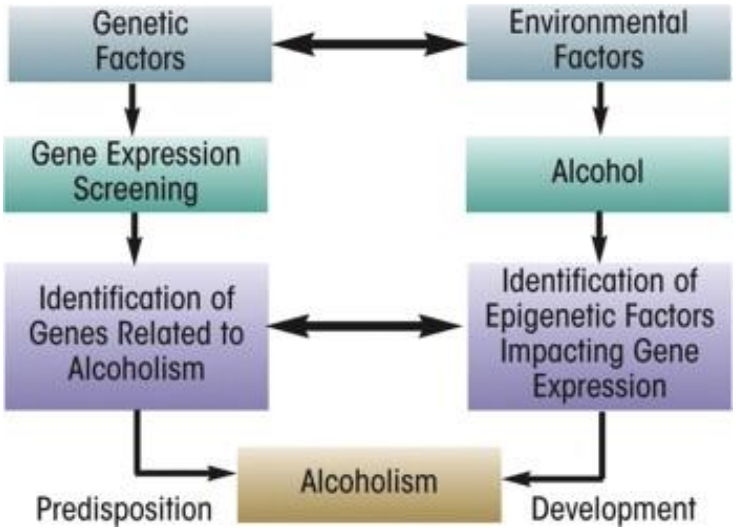


FIGURE 1.8: THE DIFFERENT FACTORS INFLUENCING ALCOHOLISM

Alcoholism is a complex disease caused by genetic and environmental factors. The neural changes characterized by alcoholism result from a complex dynamic system with a plethora of contributing factors. These factors are both environmental and internal. These internal factors depend on complex genetic states and molecular interactions. Since the normal brain is one of recursive feedback loops and regulatory controls, a drug like alcohol (whose effects are global) demonstrates radical alterations with a variety of entry points. Articulating the causes of alcoholism thus relies on research at a variety of scales and modalities. (Adapted from Starkman et al., 2012.)

Once a person becomes an alcoholic, drinking becomes increasingly compulsive and seems to escapes voluntary control. The heterogeneous nature of alcoholism reflects

the complex and multifaceted nature of alcohol's molecular effects on the CNS. The fact that multiple neurotransmitter systems are affected by alcohol makes it difficult to pinpoint the molecular mechanisms that are primarily responsible for the disease. Indeed, alcohol's molecular targets are many and varied, and include modifications of the genome, transcriptome, and proteome. Alcoholism is most likely the result of the cumulative interactions within and between all three systems. Nevertheless, identifying the molecular targets is a crucial step in understanding alcoholism and developing new treatments.

MicroRNAs are one type of molecule that is clearly involved in the neuroadaptive responses induced by exposure to substances of abuse, and their large number of targets encompasses a dynamic regulatory network. However, the processes by which microRNAs and mRNAs target cellular and synaptic function are not well-understood. Because a single microRNA targets many mRNAs, drugs of abuse can effectively hijack a complex network. Indeed, the biological pathways that have been mentioned here are diverse and indicative of the complex disease states associated with drugs of abuse. Identification of the important RNA signaling systems involved in drug dependence provides new areas of focus for therapeutic interventions.

The diverse mRNAs and neuroadaptations associated with drug dependence may be controlled by some common microRNAs. Also, a subset of the mRNA changes within a single disease state may be driven by even a smaller number of microRNAs, underscoring the potential impact of finding those key molecules. While microRNAs may be crucial for regulating synaptic plasticity, a pivotal neuroadaptation in addictive behaviors, we must also understand their role in mediating a variety of context-dependent behaviors.

In the rest of this thesis, I identify the alcohol-responsive synaptic mRNAs as a result of chronic alcohol use and determine whether they are different in the synapse

compared to the cell body (chapter 2). I then focus on identifying the alcohol-responsive synaptic microRNAs and predicting their regulatory interactions with synaptic mRNAs (chapter 3). Lastly, I manipulate these key microRNAs in-vivo to reverse and prevent alcohol consumption and alcohol-induced-neuroadaptations (chapter 4).

In the first experimental section (chapter 2), I show that chronic alcohol use causes persistent changes in networks of synaptic mRNAs, and that this effect may be mediated by microRNAs localized in the synapse. In the second experimental section (chapter 3), I describe a subset of alcohol-responsive microRNAs and predict which alcohol-responsive mRNAs they may be targeting, providing a list of key microRNA-mRNA interactions. In the final experimental section of this thesis (chapter 4), I focus on manipulation of synaptic microRNAs and demonstrate that a change in expression of one specific synaptic microRNA, miR-411, can cause a decrease in alcohol consumption and preference in chronically consuming mice, without affecting total fluid intake or saccharin consumption, and without changing acquisition of alcohol consumption in naïve mice. Furthermore, manipulation of miR-411 in alcohol consuming mice did not induce anxiolysis nor locomotion, as measured with elevated plus maze and open field test. We show that these effects were caused through changes in expression of a predicted protein, providing a mechanism by which interaction between synaptic microRNAs and alcohol can affect proteins, synaptic structure and ultimately, behavior.

The role of individual microRNAs in discrete cellular compartments underscores their essential role in cellular function and the widespread impact that drugs of abuse can exert by targeting microRNAs. This thesis describes the first study to target synaptic mRNA, such that the microRNA regulation over mRNA will take place in the synapse, having a larger impact on synaptic plasticity. Results use as proof of concept that synaptic gene networks may yield viable candidates for manipulation of behavior. Findings impact

the basic and translational science of drug dependence and prevention, spurring the development of new treatments.

CHAPTER 2: THE SYNAPTONEUROSOME TRANSCRIPTOME: A MODEL FOR PROFILING THE MOLECULAR EFFECTS OF ALCOHOL

The work presented in this chapter has been published in 2015 in the Pharmacogenomics Journal. Most D, Ferguson L, Blednov Y, Mayfield RD, Harris RA. The synaptoneurosome transcriptome: a model for profiling the molecular effects of alcohol. 2:177-88. PubMed PMID: 25135349. Supplementary material to this work can be found in the online version of the Manuscript at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4334750/#SD2.

Abstract

Chronic alcohol consumption changes gene expression, likely causing persistent remodeling of synaptic structures via altered translation of mRNAs within synaptic compartments of the cell. We profiled the transcriptome from synaptoneurosomes (SNs) and paired total homogenates (THs) from mouse amygdala following chronic voluntary alcohol consumption. In SN, both the number of alcohol-responsive mRNAs and the magnitude of fold-change were greater than in the THs, including many GABA-related mRNAs upregulated in SNs. Furthermore, SN gene co-expression analysis revealed a highly connected network, demonstrating coordinated patterns of gene expression and highlighting alcohol-responsive biological pathways, such as long-term potentiation, long-term depression, glutamate signaling, RNA processing and upregulation of alcohol-responsive genes within neuroimmune modules. Alterations in these pathways have also been observed in the amygdala of human alcoholics. SNs offer an ideal model for detecting intricate networks of coordinated synaptic gene expression and may provide a unique system for investigating therapeutic targets for the treatment of alcoholism.

Introduction

Alcohol dependence is a severe and widespread disease. Over 17 million Americans suffer from alcohol-related problems; total cost estimates of substance abuse in the United States exceed $600 billion annually, with 39% of that cost related to alcohol

(Rehm, Mathers et al. 2009). The pharmacotherapies available today are significantly limited due to side effects and failure to relieve drug craving, leading to high relapse rates.

Chronic alcohol use produces long-term neuroadaptations in synaptic structure and function, which are likely caused by persistent changes in gene expression (Rimondini, Arlinde et al. 2002, Arlinde, Sommer et al. 2004, Liu, Lewohl et al. 2006, Melendez, McGinty et al. 2012). This leads to a remodeling of neural circuitry (Mayfield, Lewohl et al. 2002, Kerns, Ravindranathan et al. 2005, Hansson, Rimondini et al. 2008) and is one of the main features of addiction (Nestler 2001, Kauer and Malenka 2007). Synaptic translation of mRNA is a cardinal process underlying normal synaptic functions (Raab-Graham, Haddick et al. 2006, Zhu, Cao et al. 2011), and perturbation by alcohol represents a mechanism contributing to synaptic neuroadaptations (Barak, Liu et al. 2013). The composition of specific mRNAs in the synaptic compartment may give insight into the neurobiology of different states of addiction and is an unexplored avenue of research.

Given the role of synaptic plasticity in alcohol dependence, selecting a biologically relevant system for analyzing the synaptic transcriptome is of critical importance. Although total homogenate (TH) preparations have been used for mRNA and alcohol studies in the past, this method limits identification of regional mRNAs and likely underestimates the number and magnitude of alcohol-responsive transcripts in the synapse. Synaptoneurosomes (SNs) contain membrane vesicles of presynaptic and postsynaptic compartments composed of primarily neurons as well as astrocytes and microglia. SNs have been used to study local translation of mRNAs in the synapse (Raab-Graham, Haddick et al. 2006) and may prove to be a superior model system for alcohol effects confined to synaptic regions of the cell.

In order to measure discrete changes within the synaptic transcriptome following chronic alcohol consumption, we profiled mRNAs from SN (Hollingsworth, McNeal et al. 1985, Sung, Weiler et al. 2004, Raab-Graham, Haddick et al. 2006) and TH samples from mouse amygdala, a brain region known to be involved with the negative reinforcement of alcohol and other drugs of abuse. The present findings reveal greater expression of alcohol-responsive mRNAs in SN compared with TH. Using gene expression patterns to generate biological networks, the SN preparation appears ideally suited for detecting alcohol-responsive groups of genes that have been shown to be important in human alcoholism. The gene clusters isolated in SN could prove useful in developing targets for the future treatment of alcoholism.

Materials and Methods

Animal Housing and Alcohol Self-Administration

Adult (2-month old) C57BL/6 J female mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were maintained at the University of Texas at Austin Animal Research Center. Mice were given a one-week acclimation period in combined housing and another week to acclimate to the bottle position in individual housing. Food and water were provided ad libitum and monitored daily, as were the temperature and light/dark cycles. Mice underwent a 30-day two-bottle choice paradigm with continuous (24 h) access to one bottle of 20% ethanol and one bottle of water, similar to that described previously (N=8 alcohol group, N=13 control group). Bottle weights were recorded daily, and the amount of alcohol consumed throughout the 30 days was calculated as g/kg (Supplementary Figure S1). Bottle positions were changed daily to control for position preferences, and mice were weighed every 4 days. All procedures were approved by the Institutional Animal Care and Use Committee at the

University of Texas at Austin and adhere to NIH Guidelines for the ethical care and use of animals in research.

SN Preparation and RNA Extraction

Mice were euthanized by cervical dislocation and then decapitated. Brains were removed and washed for 1 minute with 1 ml of ice-cold Homogenizing Buffer (HB) containing 20 mM Hepes, 1 mM EDTA (pH 7.4), 40 U/ml RNAseOut (Invitrogen, Carlsbad, CA, USA), phosphatase inhibitor cocktail 3 (Sigma, St Louis, MO, USA) and protease inhibitors 'Complete' (Roche, Indianapolis, IN, USA). Brains were then placed in a coronal Zivic mouse brain slicer with a 0.5 mm resolution (Zivic Instruments, Pittsburgh, PA, USA) and sliced in the following coordinates in order to isolate extended amygdala (two coronal slices were made for greater ease of dissection): coronal level 56–66 (Bregma (−0.18)–(−1.155)) and 66–80 (Bregma (−1.155)–(−2.55)). The extended amygdala was dissected, placed in ice- cold HB (250 ml) and homogenized for 1 minute using a VWR homogenizer and pestle (VWR, Radnor, PA, USA). To minimize homogenate loss, pestles were washed with 50 ml HB after use, and the wash was collected and added to the sample. Ten percent of the homogenate (30 ml) was snap-frozen in liquid nitrogen and stored at − 80 °C for subsequent RNA TH analysis.

Paired SNs (Hollingsworth, McNeal et al. 1985) were isolated from the rest of the homogenate (270 ml) in a manner similar to that described previously (Raab-Graham, Haddick et al. 2006, Smalheiser 2008, Sosanya, Huang et al. 2013). Briefly, homogenates were filtered through a 100-µm pore filter and subsequently through a 5-µm pore filter (Millipore, Billerica, MA, USA); filters were washed with HB before use for protection from RNAse. To maximize yield, the filters were washed with 50 ml HB after use, and the wash was collected and added to the homogenate. The homogenate was then centrifuged at

14,000g for 20 minutes at 4 °C in order to pellet the cell fraction containing SNs (Raab-Graham, Haddick et al. 2006, Sosanya, Huang et al. 2013). The supernatant was removed and the pellet snap-frozen and stored at −80 °C for SN RNA analysis. Microscopy was used to further characterize the SN preparation (see Supplementary Methods).

Total RNA was extracted from 21 SN and 21 paired TH samples with the Direct-Zol RNA extraction kit (Zymo Research Corporation, Irvine, CA, USA), using IC columns according to the manufacturer's instructions. The RNA was quantified using NanoDrop 1000 (Thermo Fisher Scientific Inc., Rockford, IL, USA) and assayed for quality using Agilent 2100 Tape- Station (Agilent Technologies, Santa Clara, CA, USA). The cutoff criteria were set on 280/260>1.7, RIN>6.5 and amount of total RNA>500 ng.

Microarray Hybridization, Data Quality Assessments and Analysis

RNA samples were processed at the University of Texas Southwestern Medical Center microarray facility in Dallas. MRNA was amplified and biotin-labeled using the Illumina TotalPrep RNA Amplification kit (Ambion, Austin, TX, USA) and hybridized to Mouse WG-6 v2.0 Expression BeadChips (Illumina, San Diego, CA, USA). Each array contained SN and paired TH samples from control and alcohol-treated mice. These were assigned randomly to each array. The array data were analyzed using R environment and Bioconductor packages, similar to our published studies (Ponomarev, Wang et al. 2012, Osterndorff-Kahanek, Ponomarev et al. 2013). The 'Lumi' package (Du, Kibbe et al. 2007, Du, Kibbe et al. 2008, Lin, Du et al. 2008) was used to preprocess the data using variance stabilization transformation (variance within array) quantile normalization (variance between arrays) and background subtraction (Dunning, Ritchie et al. 2008, Lin, Du et al. 2008). Quality measures were taken before and after preprocessing using the arrayQualityMetrics package (Kauffmann, Gentleman et al. 2009, Kauffmann and Huber

2010) to remove outliers determined by at least two out of the three tests in the package, and care was taken that the normalization did not skew the data. This package was also used to generate the principal component analysis. Transcripts significantly detected on 80% of the arrays were used in the analysis (detection probability <0.05). The data presented in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar, Domrachev et al. 2002) and are accessible through GEO Series accession number GSE51730.

The 'Limma' package (Smyth 2005) was used for differential expression analysis between SN and paired TH samples (paired/dependent t-test) and between the alcohol and control samples in SN and TH (two independent t-tests). A list of alcohol-responsive mRNAs was compiled from the list of genes differentially expressed between alcohol and control samples. A weighted gene correlation (co-expression) network analysis was generated for the combined control and alcohol data, using the weighted gene correlation (co-expression) network analysis (WGCNA) package (Langfelder and Horvath 2008). Alcohol-responsive mRNA enrichment analysis was performed for each module using an over-representation (hypergeometric) test with a cut off $P<0.05$. To determine alcohol-responsive SN and TH modules, we used the 'alcohol-responsive mRNAs' lists from our data. For details on the WGCNA parameters, see Supplementary Methods. We evaluated whether the correlation between alcohol consumption and TH modules would increase depending on WGCNA parameters. We generated another TH WGCNA network and optimized for the highest correlation of modules with consumption (top 10% of the modules). Enrichment and clustering analyses were performed using KEGG pathways, Wikipathways, gene ontologies and protein interactions, part of the Database for Annotation Visualization and Integrated Discovery (DAVID) (Huang, Sherman et al. 2009, Huang, Sherman et al. 2009), WEB-based GEne SeT AnaLysis Toolkit (Webgestalt) (Zhang,

Kirov et al. 2005, Wang, Duncan et al. 2013) and Ingenuity Pathway Analysis (IPA; Qiagen, Valencia, CA, USA). All P-values from these analyses were adjusted using the Benjamini–Hochberg method (BH). Synaptic mRNA enrichment was assessed using a list of mRNAs enriched in the synaptic neuropil and in process-localized mRNAs (Cajigas, Tushev et al. 2012). For alcohol-responsive mRNA enrichment, we used a human alcoholic mRNA data set (Ponomarev, Wang et al. 2012) from the amygdala, quantitative trait loci mRNA list (Mulligan, Ponomarev et al. 2006) and a list of mRNAs from prefrontal cortex of C57BL6 after a two-bottle choice paradigm (Osterndorff-Kahanek, Ponomarev et al. 2013). For cell types and immune response enrichment, we used the following lists of genes: neuronal, astrocytic and oligodendrocyte (Cahoy, Emery et al. 2008), microglial (Oldham, Konopka et al. 2008), glutamate/GABA (Sugino, Hempel et al. 2006) and lipopolysaccharide (LPS)-regulated mRNAs (Osterndorff-Kahanek, Ponomarev et al. 2013).

Results

The SN Transcriptome is Composed of Synaptic mRNAs and is Distinct from the TH

We compared SN and TH transcriptomes from mouse amygdala and detected 17,514 and 18,318 transcripts in the SN and TH microarrays respectively, with a high overlap of detected transcripts (17,265). We studied the expression levels using principal component analysis and found a distinct clustering of the two types of preparations, while showing a homogenous sample population within each preparation (Figure 2.1A). The clustering was evident along the first principal component, indicating that the largest variation stems from distinct expression levels in the two preparations. We identified 4,539 differentially expressed unique mRNAs (BH, $P<0.05$), with 2,119 mRNAs enriched in

the SN (Supplementary Table S1) and 2,420 mRNAs enriched in the TH (Supplementary Table S2). We used DAVID to compare the SN- and TH-enriched cellular components and found that the SN contained fewer somatic and intracellular components, while preserving and enriching the synaptic mRNAs (Table 2.1).

DAVID Enrichment Clustering	SN Score	TH Score
Intracellular	20 (1370)	32.4 (1528)
Organelle	6.2 (438)	29.7 (621)
Organelle Membrane	3.5 (137)	13.2 (179)
Synapse	2.9 (64)	1.3 n.s. (21)

TABLE 2.1: COMPARISON OF THE DAVID ENRICHMENT SCORES OF SN- AND TH-ENRICHED CELLULAR COMPONENTS

Abbreviations: The table illustrates reduction of somatic and intracellular components and preservation/enrichment of synaptic mRNAs in SN (the number of genes detected in a cluster are shown in parenthesis). All scores for enrichment clustering are significant with a BH $P<0.05$. A n.s. score was defined as a cluster containing only one significant group out of five. DAVID, Database for Annotation Visualization and Integrated Discovery; n.s., non-significant.

Among the SN-enriched transcripts, many known synaptic mRNAs were over-represented in this preparation (Table 2.2).

Annotation Cluster	DAVID Enrichment Score	Number of mRNAs	P-value	BH P-value	Gene Symbols
Regulation of system process	4.1	18	8.60E-06	1.60E-02	KCNMA1, MYO6, GNAI2, GRIK5, CTNND2, MECP2, GJA1, ATP1A2, CSPG5, ADORA1, RIMS1, PTPN11, HDAC4, SLC1A3, NTRK2, HOPX, DLG4, CAMK2A.
Synapse	4.0	26	4.00E-07	1.20E-04	GRIK5, TIMP4, RIMS1, ADORA1, SLC1A2, GP1BB, SNPH, DLG4, CAMK2A, DLG2, MT3, KCNMA1, PHACTR1, ARC, MYO6, DLGAP3, SPARCL1, PSD3, SSPN, SHANK3, PPP1R9B, HDAC4, NTRK2, VAMP3, UNC13C, SNTA1.
PDZ/DHR/GLGF	3.8	14	4.10E-05	3.40E-02	SNX27, PREX1, PDLIM4, PDLIM2, MPP6, SLC9A3R1, RIMS1, SHANK3, PPP1R9B, MAST2, SIPA1L1, DLG4, DLG2, SNTA1.
Cytoskeleton	3.8	51	3.30E-05	2.00E-03	KIF23, KIFC2, GFAP, TUBB2B, AIF1, FERMT2, PDLIM2, ADORA1, CTNNB1, NDE1, EVI5, DLG4, DLG2, ARC, MYO6, INPPL1, KIF5A, KIF5C, PSD3, SPIRE1, MID1IP1, TBCEL, RB1, DNAIC1, CTNNA1, FMN2, KIF1A, MAST2, KIF1B, PDE4DIP, ADD3, CAPZB, LLGL1, KLC1, GP1BB, STRBP, CDC42EP4, ACTB, DLGAP3, CKAP5, CSRP1, COTL1, SIRT2, SHANK3, PTPN11, EPB4.1L2, PPP1R9B, HDAC4, EPB4.1L1, NTRK2, SNTA1.
Transmission of nerve impulse	3.0	18	3.90E-05	2.40E-02	KCNMA1, SCD2, MYO6, ALDH5A1, GRIK5, MECP2, TIMP4, ATP1A2, ADORA1, CTNNB1, MBP, ATXN1, KIF1B, ABAT, LGI4, UNC13C, NCAN, DLG2.

TABLE 2.2: FUNCTIONAL CLUSTERING OF SN-ENRICHED MRNAS

446 mRNAs were enriched in the SN and there were 163 functional clusters. In order to find the most synaptically-enriched pathways, we used a higher threshold fold-change of 25%. The top 5 clusters are shown (BH, P<0.05). Gene symbols are shown for each cluster.

Most of these synaptic functional groups were not detected in the TH, indicating that enriched synaptic mRNAs are more readily detected in the SN. Webgestalt was used to investigate known pathways (KEGG and Wikipathways; Supplementary Table S3) and to generate a network of known protein interactions enriched in the SN (Figure 2.1B).

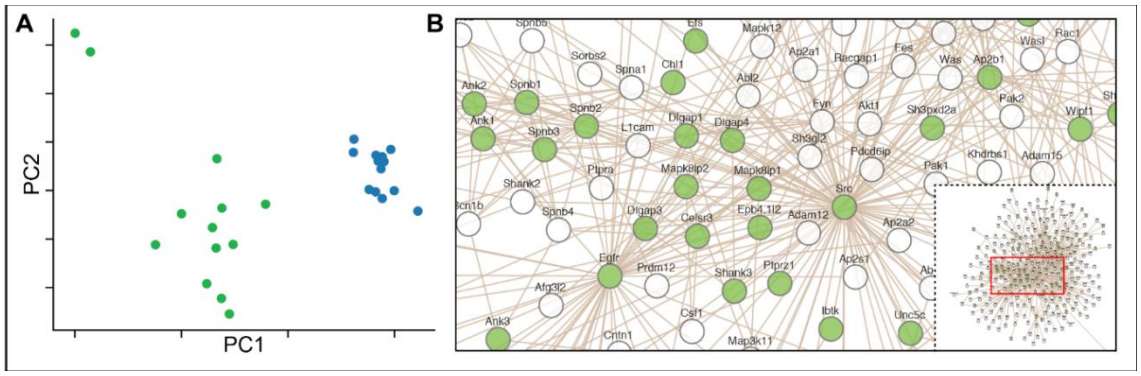


FIGURE 2.1: THE SN TRANSCRIPTOME IS COMPOSED OF SYNAPTIC MRNAS AND IS DISTINCT FROM THE TH

A. Principal component analysis of expression profiles from paired SN (green) and TH (blue) samples. Preparation difference is the first principal component and explains 17% of the variance. B. SN-enriched mRNA network illustrating known protein interactions between the SN-enriched mRNAs. The bottom right portion of the figure is an overview of the entire network, and the highlighted portion of this network has been enlarged. The green nodes represent the mRNAs enriched in the SN compared with the TH preparation (fold-change threshold of >10%, Benjamini–Hochberg method P<0.05). Many known synaptic mRNAs are found in the center of this network, emphasizing enrichment of the synaptic components in the SN preparation.

The network was associated with axon guidance and cell leading edge and highlighted the Dlg family (also known as postsynaptic density proteins or PSDs). SN (but not TH) transcripts were also over-represented with synaptic mRNAs in a high-resolution study exploring the synaptic neuropil (Cajigas, Tushev et al. 2012).

The Greater Resolution of SN Preparation Captures the Molecular Effects of Alcohol

We next identified mRNAs from the amygdala of 8 alcohol-treated and 13 control mice, for a total of 21 SN and paired TH samples. In SNs, 1,531 alcohol-responsive mRNAs were identified, compared with 462 in THs (Figure 2.2A). Examples of alcohol-responsive mRNAs, fold-changes and P-values are shown in Table 2.3, and the full list is shown in Supplementary Table S4.

Illumina ID	Gene Symbol	Gene Info	SN Correlation with Alcohol Consumption	SN Fold-Change	SN P-value	TH Fold-Change	TH P-value
ILMN_1229256	Bzrap1	benzodiazepine receptor associated protein 1	-0.48	0.80	2.00E-02	0.99	8.72E-01
ILMN_1222167	Gria2	glutamate receptor, ionotropic AMPA2 (GluA2)	-0.47	0.81	2.37E-02	1.04	5.74E-01
ILMN_2483253	Dicer1	dicer 1, ribonuclease type III	-0.54	0.86	5.71E-03	1.01	8.13E-01
ILMN_1240346	Socs5	suppressor of cytokine signaling 5	-0.62	0.86	1.14E-03	0.97	5.75E-01
ILMN_2644632	Stxbp1	syntaxin binding protein 1	-0.66	0.86	5.84E-04	1.01	8.89E-01
ILMN_1231506	Cnrip1	cannabinoid receptor interacting protein 1	-0.67	0.87	5.71E-04	1.00	9.94E-01
ILMN_3105417	Bdnf	brain derived neurotrophic factor	-0.60	0.88	2.98E-03	0.95	2.52E-01
ILMN_2622817	Kcnq2	potassium voltage-gated channel, subfamily Q, member 2	-0.46	0.88	2.77E-02	1.04	2.74E-01
ILMN_3061460	Ntrk2	neurotrophic tyrosine kinase, receptor, type 2	-0.42	0.88	4.04E-02	0.98	5.22E-01
ILMN_2724044	Sncb	synuclein β	-0.49	0.89	3.41E-02	1.02	6.21E-01
ILMN_2760927	Kcna6	potassium voltage-gated channel, shaker-related, subfamily, member 6	-0.47	0.90	2.25E-02	0.97	5.43E-01
ILMN_2713841	Hspd1	heat shock protein 1 (chaperonin)	0.47	1.10	4.01E-02	0.97	5.63E-01
ILMN_1217180	Ifitm1	interferon induced transmembrane protein 1	0.46	1.11	2.96E-02	1.09	1.41E-01
ILMN_1242178	Adh5	alcohol dehydrogenase 5 (class III), chi polypeptide	0.46	1.15	2.65E-02	0.99	7.31E-01
ILMN_2733179	Aldh2	aldehyde dehydrogenase 2, mitochondrial	0.46	1.15	2.76E-02	0.96	5.25E-01
ILMN_1214715	Gfap	glial fibrillary acidic protein	0.49	1.16	1.31E-02	1.03	4.81E-01
ILMN_1231625	Cyp4f14	cytochrome P450, family 4, subfamily f, polypeptide 14	0.62	1.17	2.82E-03	1.01	6.59E-01
ILMN_2771956	Calu	Calumenin	0.60	1.17	3.00E-03	1.03	1.21E-01

Illumina ID	Gene Symbol	Gene Info	SN Correlation with Alcohol Consumption	SN Fold-Change	SN P-value	TH Fold-Change	TH P-value
ILMN_2881480	Vamp3	vesicle-associated membrane protein 3	0.54	1.17	1.16E-02	0.95	8.43E-02
ILMN_2945095	Tnfrsf10b	tumor necrosis factor receptor superfamily, member 10b	0.49	1.18	1.63E-02	1.03	5.29E-01
ILMN_1229720	Tollip	toll interacting protein	0.44	1.18	2.09E-02	1.07	9.35E-02
ILMN_2719908	Cyp2j9	cytochrome P450, family 2, subfamily j, polypeptide 9	0.49	1.18	2.03E-02	1.06	6.38E-02
ILMN_2705777	Gstm5	glutathione S-transferase, mu 5	0.61	1.20	2.32E-03	1.02	7.91E-01
ILMN_1255438	Cpped1	calcineurin-like phosphoesterase domain containing 1	0.71	1.20	2.65E-04	1.00	9.80E-01
ILMN_2760800	Cxcl14	chemokine (C-X-C motif) ligand 14	0.53	1.20	9.84E-03	1.03	6.64E-01
ILMN_2680745	Gabbr1	γ-aminobutyric acid (GABA) B receptor, 1	0.40	1.20	3.23E-02	1.00	9.82E-01
ILMN_2776008	Gstk1	glutathione S-transferase kappa 1	0.52	1.21	1.36E-02	0.99	7.63E-01
ILMN_2619620	C1qb	complement component 1, q subcomponent, β polypeptide	0.45	1.21	3.30E-02	0.99	8.93E-01
ILMN_3149251	Glud1	glutamate dehydrogenase 1	0.40	1.23	3.84E-02	1.07	2.70E-01
ILMN_1239110	Eef2	eukaryotic translation elongation factor 2	0.47	1.42	1.48E-02	1.08	2.69E-01

TABLE 2.3: THIRTY ALCOHOL-RESPONSIVE MRNAS IN SN THAT WERE NOT SIGNIFICANTLY CHANGED IN TH

SN mRNA correlation with alcohol consumption, SN and TH treatment fold-change, and p-values are shown. Fold-change >1 indicates an increase in expression and fold-change <1 indicates a reduction.

We examined the number and magnitude of fold-changes (Figure 2.2B) as a potential means for identifying the most important mRNAs involved in alcohol-induced changes. SNs had three times more alcohol-responsive mRNAs and larger fold-changes

than THs. 23% percent of the TH alcohol-responsive mRNAs were also detected in SN, compared with only 7% of SN alcohol-responsive mRNAs that were detected in THs (Figure 2.2C).

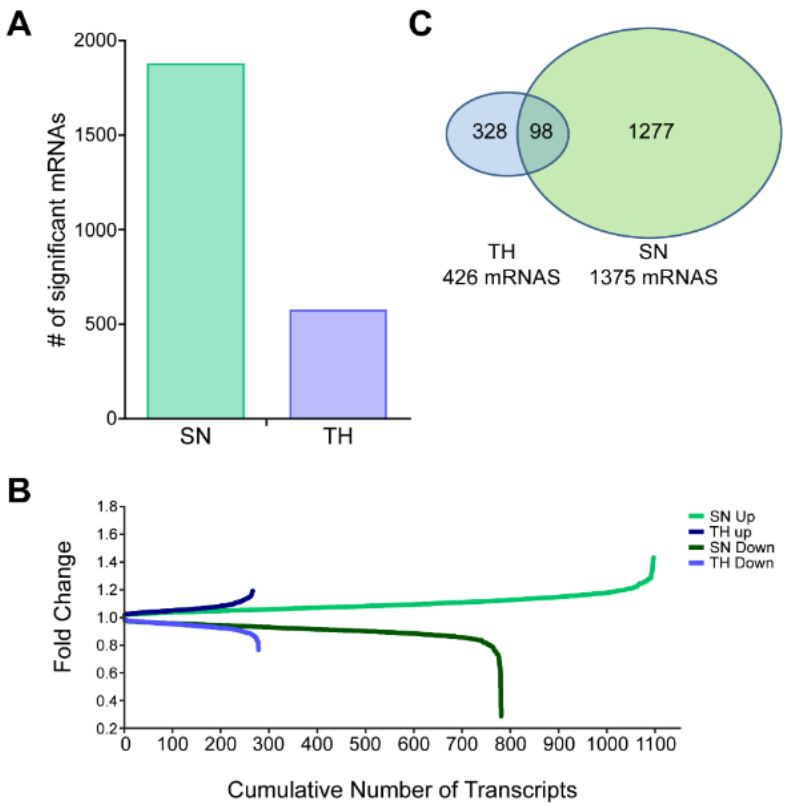


FIGURE 2.2: THE GREATER RESOLUTION OF SN PREPARATION CAPTURES THE MOLECULAR EFFECTS OF ALCOHOL

A. The number of alcohol-responsive annotated mRNAs identified in paired SN and TH samples for P<0.05 are shown (N=8 for alcohol and N=13 for control). B. Fold-change produced by alcohol consumption is shown as a function of the cumulative number of transcripts. Alcohol-induced changes in the number of transcripts and magnitude of fold-changes. C. Venn diagram showing the overlap in alcohol-responsive unique mRNAs between the SN and TH preparations (P<0.05).

A functional annotation of the top alcohol-responsive mRNAs revealed a higher enrichment score for synaptic mRNAs in SNs than in THs. IPA was used to study the molecular and cellular functions of SN alcohol responsive mRNAs, highlighting the

following top five pathways: molecular transport, protein trafficking, RNA posttranscriptional modification, cell morphology, and DNA replication, recombination, and repair. Importantly, the alcohol-responsive mRNA list from SNs was strikingly similar to the mRNA list obtained from human alcoholics (Table 2.4), suggesting that the alcohol-drinking paradigm used in this study induced similar mRNA changes in mouse amygdala to that observed in human amygdala (Ponomarev, Wang et al. 2012) and also demonstrating that SNs are ideal for characterizing transcriptome changes in chronic ethanol-treated mice that are relevant in human alcoholics. The SN alcohol-responsive mRNAs were highly enriched for neuron process-localized mRNAs (Cajigas, Tushev et al. 2012) and contained many alcohol-consumption quantitative trait locus genes found in mice (Mulligan, Ponomarev et al. 2006), further highlighting this preparation as a tool to detect alcohol-responsive mRNAs related to synaptic function and structure.

	Human Alcohol-Responsive Genes		Cell Process mRNAs		Alcohol QTL Genes	
	Number of mRNAs	P-value	Number of mRNAs	P-value	Number of mRNAs	P-value
SN Alcohol-Responsive mRNAs	327	1.39E-04	1107	3.07E-03	358	8.74E-02
TH Alcohol-Responsive mRNAs	83	7.59E-01	358	7.54E-02	114	2.77E-01

TABLE 2.4: OVER-REPRESENTATION OF HUMAN ALCOHOLIC MRNA, PROCESS MRNAS AND QTL GENES

Abbreviation: QTL, quantitative trait loci. The significant over-representations are highlighted in pink.

Alcohol Affects the Transcriptome in a Coordinated Manner

We used WGCNA (Langfelder and Horvath 2008) to group genes into modules that have strong co-varying (similar) patterns of expression across the sample set.

Hierarchical clustering showed that the SN preparation contains groups of mRNAs that have highly coordinated patterns of expression (Figure 2.3A). When using the same parameters for THs, the majority of mRNAs showed dissimilar levels of expression (marked by the gray color) (Figure 2.3B).

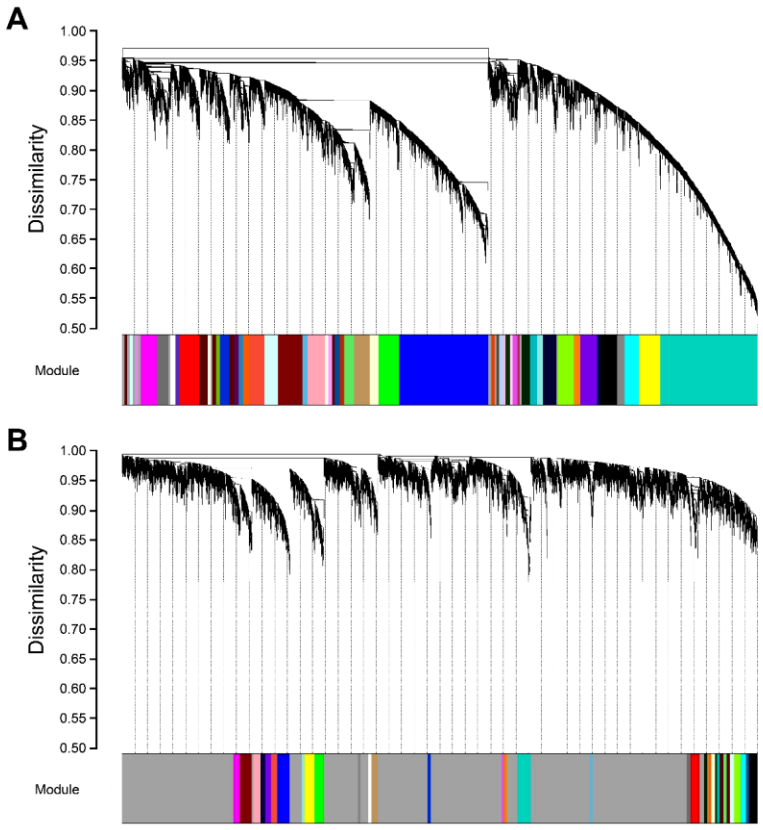


FIGURE 2.3: ALCOHOL AFFECTS THE TRANSCRIPTOME IN A COORDINATED MANNER AS SEEN IN SN

A. Results of a dendrogram-hierarchical cluster of mRNAs from SN (N=21). In the cluster, each end point represents a gene, and the genes are arranged by similarity in covariance. Genes under the same branch of the dendrogram are more similar than those outside of the branch, and their dissimilarity is represented by the y axis. Gray represents genes unrelated to others. SN preparation contains groups of mRNAs that have highly coordinated patterns of expression as seen by the low dissimilarity values. B. Results of a dendrogram-hierarchial cluster of mRNAs from TH (N=21). High dissimilarity values (marked by the gray color) indicate that the TH contains fewer mRNAs with similar patterns of expression.

We adjusted the TH network parameters to optimizing module correlation with alcohol consumption. Allowing for less similarity between the mRNAs would enable greater detection of group-clustered modules in THs (Supplementary Figure S3A). However, the change in parameters did not significantly affect the correlation between the TH modules and alcohol consumption (Supplementary Figure S3B). In SNs, 40% of the modules were significantly correlated with alcohol consumption (average r=0.6, P<.05) (Supplementary Figure S3C). We then determined the modules that were overrepresented with alcohol-responsive mRNAs (referred to as 'alcohol-responsive modules'). In SNs, 10 out of the 54 alcohol responsive modules were detected (8 were upregulated and 2 were downregulated; Supplementary Table S5).

These modules were significantly associated with biological pathways such as pathways for long-term potentiation and depression and RNA processing, and contained many mRNAs associated with potassium channels, glutamate and GABA systems. Upregulated mRNAs include Camkk2, Camta1, Capn2, Ntrk2, Ntsr2, Stx18, Stx8, Stxbp4, Syap1, Synj2bp, Prkcdbp and Grk6. Downregulated mRNAs include brain-derived neurotrophic factor (BDNF), Camsap3, Capn6, Negr1, Nptn, Ntrk2, Unc5c, Stx3, Stxbp1, Stxbp2, Syncrip, Sst, Sstr2, Sncb and Timp4. The potassium channel family was also highly responsive to alcohol and includes the voltage-gated potassium channels (Kcna6 and Kcnq2, downregulated), calcium-activated Kcnu1 (SLO-3-slowpoke3, downregulated) and inwardly rectifying potassium channels (Kcnj1 and Kctd20, upregulated). The following glutamate- and GABA-related transcripts were upregulated: Grk6, Glud1, Slc1a2, Slc1a3, Gabbr1, and Gabrb2, whereas the following were downregulated: Grina, Gria2, Grip1, VGlut2 (Slc17a6), Grm7, and Narg2.

As mentioned, RNA processing machinery was a highly overrepresented biological pathway associated with alcohol-responsive mRNAs (Figure 2.4). These mRNAs include

RNA transcriptional, translational, spliceosomal and editing machineries, as well as many mRNAs for ribosomal proteins, suggesting that chronic alcohol affects translational mechanisms in the synapse.

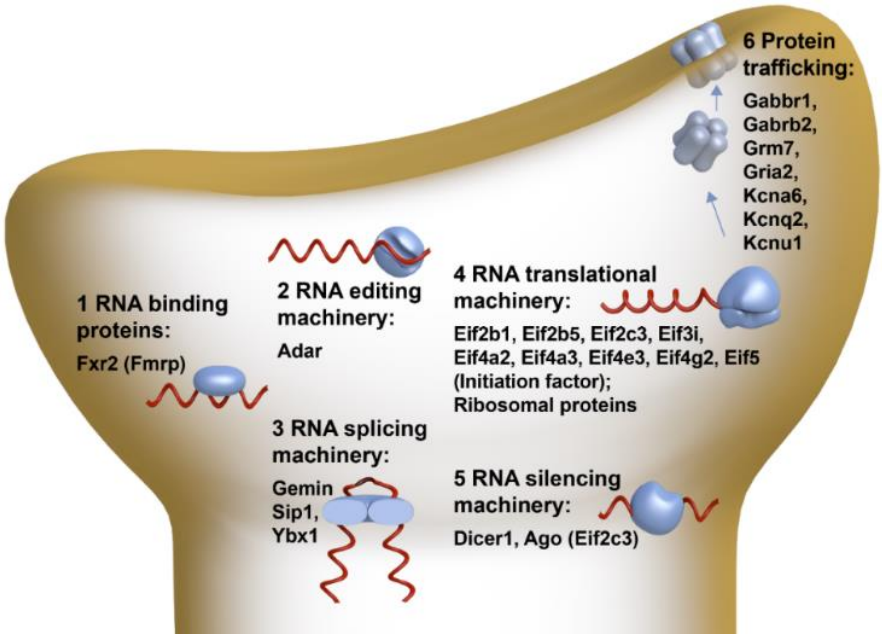


FIGURE 2.4: ALCOHOL REGULATES RNA PROCESSING AND TRANSLATIONAL MACHINERY IN THE SYNAPSE

Examples of alcohol-responsive mRNAs related to known RNA processing pathways, illustrated on a postsynaptic compartment of a neuron, which was enriched in the synaptoneurosome preparation.

SN Alcohol-Responsive Effects are Cell-Type Specific

A cell-type-specific enrichment analysis was performed for the alcohol-responsive mRNAs using neuronal, astrocytic, oligodendrocyte, microglial, GABA and glutamate gene lists (see Methods for details). The upregulated alcohol-responsive mRNAs in SNs were enriched with microglial, astrocytic and GABAergic cell types, while the downregulated mRNAs were enriched in neuronal cell types (Table 2.5). This trend of upregulation of microglial cell types was also true for SN modules in the WGCNA network (Figure 2.5A).

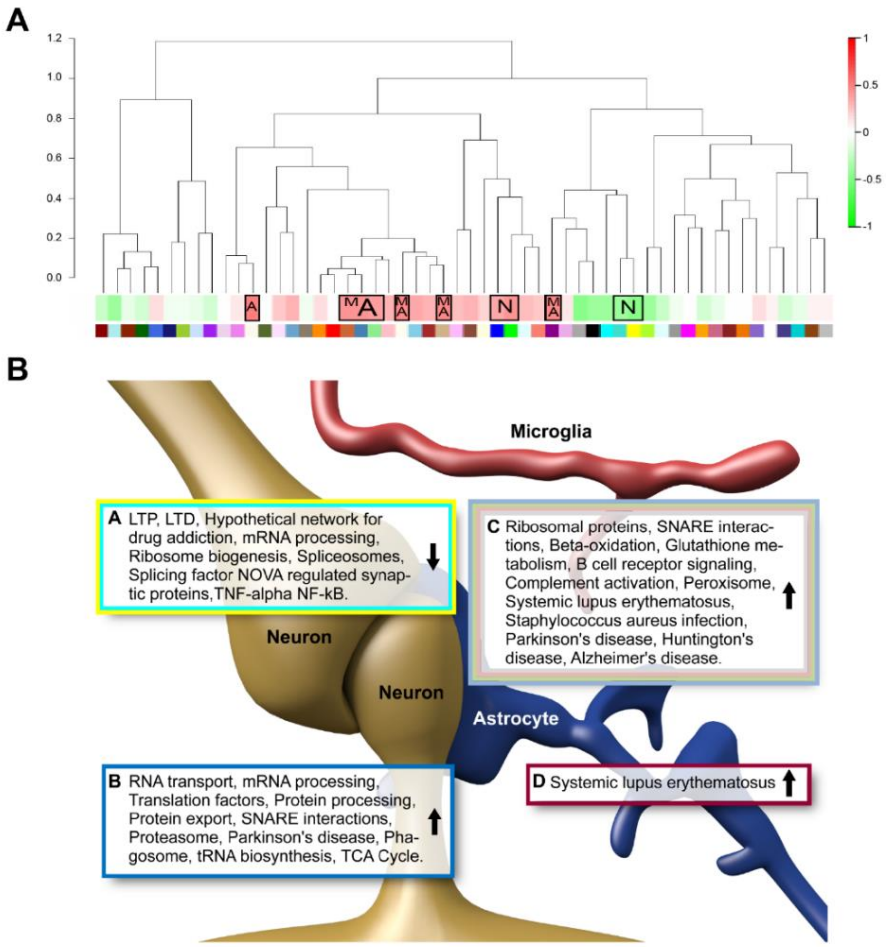


FIGURE 2.5: SN ALCOHOL-RESPONSIVE EFFECTS ARE CELL-TYPE SPECIFIC

A. Alcohol-responsive synaptic modules, correlation with alcohol consumption and cell specificity. The dendrogram shows the hierarchical relationship between the gene modules for the SN mRNA network. Below the dendrogram, a heat map shows the module correlation to amount of alcohol consumed; red and green indicate strong positive and negative correlations, respectively. The boxed correlations represent the significant alcohol-responsive modules (over-representation-hypergeometric test), and the letters inside the boxes represent the cell type over-represented in the modules: N=neuron, M=microglia, and A=astrocyte. The different colors under the dendrogram correspond to the different modules. B. Alcohol-responsive modules, cell specificity and biological pathways. The KEGG and Wikipathways and biological functions identified as alcohol-responsive are shown for each of the modules in SN, illustrating the most critical biological functions in groups of co-expressed mRNAs (DAVID (Database for Annotation Visualization and Integrated Discovery) and Webgestalt, BH (Benjamini–Hochberg method) corrected $P<0.05$). Pathways from modules with similar expression were grouped together for each of the over-represented cell types. The arrow in each box represents the direction of alcohol response (that is, upregulation or downregulation by alcohol treatment). The text-box border colors correspond to the module colors. Boxes A and B are enriched for neurons; Box C is enriched for microglia and astrocytes; Box D is enriched for astrocytes. Cell type illustrations were modified from Kettenman et al., 2013.

Of the eight alcohol-responsive upregulated modules in SN, six were also positively correlated with alcohol consumption and enriched with microglia and astrocyte mRNAs (Table 2.6). The two downregulated modules were negatively correlated with alcohol consumption and enriched in neuronal mRNAs. DAVID and Webgestalt were used to find the KEGG and Wikipathways over-represented in each of the modules (Figure 2.5B). In the THs, only three modules were found to be cell specific (two astrocytic/microglial and one neuronal).

Data set	Microglia	P-value	Astrocytes	P-value	Neuron	P-value	Oligodendrocytes	P-value	Glutamate	P-value	GABA	P-value
SN enriched	28	5.02E-04	250	1.03E-27	39	8.68E-01	45	6.10E-05	102	1.13E-03	152	4.71E-22
TH enriched	15	5.97E-01	43	1.00E+00	101	2.55E-13	24	7.34E-01	166	1.71E-20	74	4.88E-01
Alcohol's effect on SN	25	5.40E-02	115	5.33E-05	55	5.87E-01	19	9.94E-01	99	4.10E-01	99	1.30E-01
Alcohol's effect on TH	7	3.94E-01	26	4.95E-01	18	4.92E-01	7	8.60E-01	32	4.53E-01	27	6.46E-01
Alcohol-responsive up-regulated SN	20	5.81E-03	91	2.51E-09	8	1.00E+00	14	8.94E-01	60	4.36E-01	70	1.27E-02
Alcohol-responsive down-regulated SN	5	8.52E-01	24	9.52E-01	47	5.12E-07	5	9.95E-01	39	4.88E-01	29	8.94E-01

TABLE 2.5: OVER-REPRESENTATION OF THE SPECIFIC CELL TYPES ASSOCIATED WITH THE ALCOHOL-RESPONSIVE MRNAS IN SN AND TH

The number of overlapping mRNAs and over-representation P-values are shown for microglia, astrocyte, neuron, oligodendrocyte, glutamate and GABA mRNA lists. Significant values are ones with a $P<0.05$. Fold changes above 10% were used for the SN- and TH-enriched lists.

Module	# of significant mRNAs in module	P-value	Average Fold-Change	Microglia	P-value	Astrocytes	P-value	Neuron	P-value	Oligodendrocytes	P-value	Correlation with alcohol	Correlation P-value	KEGG pathways	Wikipathways
Tan	237	4.27E-64	1.08	11	6.52E-04	27	1.71E-05	3	1.00E+00	7	4.47E-01	0.05	8.30E-01	Oxidative phosphorylation, Metabolic pathways, Parkinson's disease, Systemic lupus erythematosus, Peroxisome, Huntington's disease, Alzheimer's disease, Valine, leucine and isoleucine degradation, Pyrimidine metabolism, Ribosome, Staphylococcus aureus infection, Purine metabolism, Proteasome	Electron Transport Chain, Complement Activation, Classical Pathway, Oxidative phosphorylation, Mitochondrial LC-Fatty Acid β-Oxidation
Ivory	39	1.88E-08	1.07	4	8.82E-03	15	3.95E-08	1	9.83E-01	3	1.90E-01	0.50	2.23E-02	Keap1-Nrf2, Glutathione metabolism, Glutathione and one carbon metabolism	Glutathione metabolism, B cell receptor signaling pathway
Blue	753	1.42E-49	1.06	18	4.59E-01	50	8.05E-01	110	2.29E-03	23	9.68E-01	-0.75	9.88E-05	Metabolic pathways, Proteasome, Oxidative phosphorylation, Protein processing in endoplasmic reticulum, Phagosome, Aminoacyl-tRNA biosynthesis, Protein export, Lysosome, Ubiquitin mediated proteolysis, Parkinson's disease, Collecting duct acid secretion, Glycolysis / Gluconeogenesis, RNA transport, Nucleotide excision repair, SNARE interactions in vesicular transport, Vasopressin-regulated water reabsorption	Proteasome Degradation, Translation Factors, mRNA processing, Oxidative phosphorylation, Electron Transport Chain, TCA Cycle
Sienna3	42	6.84E-06	1.06	2	2.60E-01	11	3.40E-04	0	1.00E+00	0	1.00E+00	0.50	1.98E-02	Systemic lupus erythematosus	NA
Lightgreen	82	9.00E-06	1.06	10	5.29E-05	24	8.66E-08	2	1.00E+00	2	9.06E-01	0.52	1.55E-02	Ribosome, Oxidative phosphorylation, Huntington's disease, Parkinson's disease, Alzheimer's disease, SNARE interactions in vesicular transport, Metabolic pathways, Peroxisome	Electron Transport Chain, Cytoplasmic Ribosomal Proteins, Oxidative phosphorylation
Steelblue	40	3.51E-03	1.05	5	5.75E-03	14	1.78E-05	1	9.97E-01	1	8.83E-01	0.38	9.06E-02	NA	NA
Green	141	1.41E-04	1.03	4	6.96E-01	26	5.56E-03	33	2.54E-02	5	9.40E-01	-0.57	7.16E-03	NA	NA

Module	# of significant mRNAs in module	P-value	Average Fold-Change	Microglia	P-value	Astrocytes	P-value	Neuron	P-value	Oligodendrocytes	P-value	Correlation with alcohol	Correlation P-value	KEGG pathways	Wikipathways
Floralwhite	24	2.08E-02	1.03	0	1.00E+00	6	2.21E-02	0	1.00E+00	0	1.00E+00	0.52	1.51E-02	Amino Acid metabolism, mRNA processing, TCA Cycle	Spliceosome, Alanine, aspartate and glutamate metabolism
Yellow	149	1.07E-04	0.96	0	1.00E+00	4	1.00E+00	49	3.63E-07	6	8.64E-01	0.32	1.57E-01	NA	Hypothetical Network for Drug Addiction, TNF-α NF-kB Signaling Pathway, PluriNetWork, mRNA processing, Splicing factor NOVA regulated synpatic proteins
Turquoise	747	4.62E-33	0.94	5	9.98E-01	15	1.00E+00	101	2.04E-05	22	7.90E-01	-0.05	8.17E-01	Long-term potentiation, Long-term depression	mRNA processing, Hypothetical Network for Drug Addiction

TABLE 2.6: OVER-REPRESENTATION OF THE SPECIFIC CELL TYPES AND BIOLOGICAL PATHWAYS ASSOCIATED WITH THE SN MODULES

The significant values (P<0.05) are marked in distinct colors per cell type. Average fold-change >1 indicates an increase in gene expression in a module and fold-change <1 indicates reduction in gene expression.

The immune system, specifically the neuroimmune system, has been recently implicated in alcohol dependence (Mayfield, Ferguson et al. 2013), and LPS treatment, which activates an immune response and enhances alcohol consumption in mice (Blednov, Benavidez et al. 2011). An over-representation analysis of the SN alcohol-responsive mRNAs with a list of LPS-regulated mRNAs showed a significant representation of LPS mRNAs ($P<0.05$) and highlighted 55 common mRNAs found in chronic alcohol and chronic LPS treatment. Many astrocyte and microglial transcripts related to neuroimmune signaling were upregulated by chronic alcohol consumption. Key immune/inflammatory genes, including Tnfaip8l2, Tnfrsf10b, Traf4 and Tollip, were all upregulated by alcohol. In addition, chemokine and complement-related transcripts were upregulated, including the following: Ccr5, C1qa, C1qb, Ccrn4l, CCR4, Cxcl14, Gfap and Gbas. The gluthathione and peroxisome pathways were altered by alcohol, almost all of which were upregulated (Gstk1, Gstm5, Gstm6, Gpt2, Gpx4, Gpx7, Pex5,Pex6, Prdx3).

Discussion

We profiled mRNAs from SNs and paired TH preparations from the amygdala of ethanol-treated mice, using a within-subject comparison, and found a robust difference between the SN and TH alcohol-responsive mRNAs. A greater number of alcohol-responsive mRNAs with larger fold-changes were detected in the SNs as well as a greater enrichment of synaptic mRNAs. Our results suggest that the SN is a useful preparation for studying synaptic (both neuronal and glial) molecular changes associated with chronic alcohol consumption.

Although there are other reports of RNA composition in synaptic preparations (Tian, Nakayama et al. 1999, Sung, Weiler et al. 2004, Poon, Choi et al. 2006, Cajigas, Tushev et al. 2012), there have been no direct comparisons of synaptic vs paired TH. The SN preparation has been used to identify synaptic networks related to neurodegenerative disorders (Williams, Shai et al. 2009), mental retardation (Zalfa, Eleuteri et al. 2007), schizophrenia (Smalheiser, Lugli et al. 2014) and

cocaine addiction (Eipper-Mains, Kiraly et al. 2011). However, this is the first alcohol study utilizing SN preparations. We used a model of alcohol consumption that produces intoxicating blood ethanol concentrations (Middaugh, Szumlinski et al. 2003) and induces mRNA expression changes in the prefrontal cortex of mice (Osterndorff-Kahanek, Ponomarev et al. 2013), as well as functional changes in the nucleus accumbens (Middaugh, Szumlinski et al. 2003). Our results, showing that the THs from amygdala contained 500 differentially expressed mRNAs, are consistent with previous findings (Osterndorff-Kahanek, Ponomarev et al. 2013). A key finding is that the chronic alcohol paradigm used here in mice induces changes in the transcriptome of the amygdala that are similar to those observed in the amygdala of human alcoholics (see Table 2.4) (Ponomarev, Wang et al. 2012). Our current results also highlight the utility of SNs compared with THs in studying alcohol's molecular effects, given that the overlapping expression changes between mouse and human were observed using SNs but not in our previous studies using THs (Osterndorff-Kahanek, Ponomarev et al. 2013).

There are two possible explanations for the difference between the SN and TH preparations. First, restricting gene expression profiling to the synaptic compartments (preventing dilution with the somatic transcriptome) should facilitate detection of specific mRNAs that are localized to the synapse. Our results from the weighted gene co-expression networks showed many mRNAs with similar or overlapping patterns of expression in SNs, while the TH network contained few overlapping networks. The similarity in functional gene networks in SNs facilitates the detection of alcohol actions that are specific to the synaptic region. Second, alcohol could selectively target synaptic mRNAs, ultimately changing gene expression in the synapse. This is supported by the finding that RNA processing machinery was responsive to alcohol. For example, RNA transcriptional, translational, spliceosomal and editing machineries, as well as many ribosomal proteins, were over-represented in the alcohol-responsive mRNAs and the different modules, suggesting that chronic alcohol use affects translation in the synapse. Studies of synaptic compartments show involvement of microRNAs in regulating synaptic translation of mRNAs (Raab-Graham, Haddick et al. 2006, Rajasethupathy, Fiumara et al.

2009). Furthermore, microRNAs, their precursors and processing enzymes show synaptic localization, suggesting a well-orchestrated microRNAs regulation in the synapse (Lugli, Larson et al. 2005, Lugli, Torvik et al. 2008, Huang, Ruiz et al. 2012). In fact, our data show that synaptic microRNAs enzymes such as Dicer1 and Eif2c3 are alcohol sensitive. Previous studies from our group anticipated that Dicer would be a predicted target of microRNAs in human alcoholic brain samples (Lewohl, Nunez et al. 2011). It is appealing to propose that alcohol affects synaptic microRNAs machinery, allowing for targeted regulation of gene expression in the synapse.

We found that BDNF and its receptor TrkB as well as potassium channels were all altered by alcohol, corroborating well-documented alcohol-induced changes in these receptors. BDNF was downregulated, whereas one TrkB transcript was downregulated and one was upregulated. These results might be due to probe hybridization differences stemming from different splice variants. BDNF is involved in synaptic plasticity (Im, Hollander et al. 2010, Lobo, Covington et al. 2010), obesity (Cao, Lin et al. 2009) and addiction (Im, Hollander et al. 2010, Lobo, Covington et al. 2010), and potassium channels have been associated with increased sensitivity and tolerance to the sedative effects of ethanol (Ghezzi, Al-Hasan et al. 2004, Cowmeadow, Krishnan et al. 2005), seizure susceptibility (Yamada, Ji et al. 2001), neonatal familial convulsions and epilepsy (Singh, Charlier et al. 1998) and may be important in the withdrawal-induced seizures caused by chronic alcohol consumption in humans.

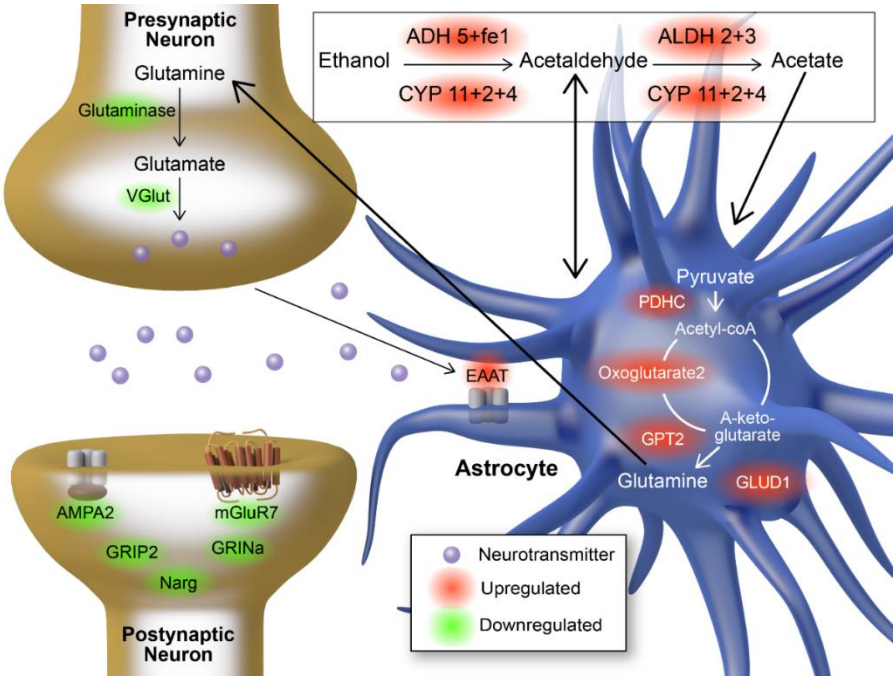


FIGURE 2.6: THEORETICAL MODEL FOR METABOLISM OF ALCOHOL IN THE BRAIN

Upregulated mRNAs are shown in red, and downregulated mRNAs are in green.

Chronic alcohol use might result in increased metabolism of alcohol or acetate in the brain (Volkow, Hitzemann et al. 1992, Wang, Volkow et al. 2000). A study in human alcoholics showed an increase in glutamate–glutamine and GABA labeling in heavy compared with light drinkers (Jiang, Gulanski et al. 2013). In our study, we found many alcohol-metabolizing enzymes that were upregulated by alcohol consumption, in agreement with a previous study (Liu, Lewohl et al. 2004). Furthermore, alcohol-responsive glutamate and GABA mRNAs were detected in SNs (GABA-related mRNAs were associated with the upregulated alcohol-responsive mRNAs, see Table 2.5). Figure 2.6 illustrates how the alcohol-responsive mRNAs can participate in alcohol degradation to produce metabolites that can enter the TCA cycle and be converted into glutamate, which may contribute to the dysregulation in the glutamate system seen in alcoholics.

The sensitivity of the SN preparation compared with the TH also allowed for improved cell-type enrichment analysis, enabling the detection of a high positive correlation of astrocyte and microglial mRNAs with alcohol consumption in the SNs. All of the alcohol responsive genes in these astrocyte/microglia modules were upregulated. Given that these cell types are generally associated with neuroinflammation, a potential consequence of chronic alcohol use is activation of neuroimmune signaling. The adaptation of the neuroimmune system is consistent with data from the amygdala of human alcoholics (Ponomarev, Wang et al. 2012) and supports the emerging concept that there is a neuroimmune response to chronic alcohol use (Mayfield, Ferguson et al. 2013). In addition, astrocytes might have role in regulating synaptic plasticity by altering the levels of glutamate, GABA and tumor necrosis factor-α available in the synapse (Pickering, Cumiskey et al. 2005, Perea, Navarrete et al. 2009). We found that all three of these systems were sensitive to alcohol, including glutamate and GABA metabolizing enzymes, receptors and transporters and tumor necrosis factor-α receptors and their interacting proteins. This suggests that the upregulated astrocyte specific genes could induce a wide range of effects following chronic alcohol consumption, ranging from neuroimmune to plasticity responses. The SN preparation also enriches for Peri-synaptic microglial and astroglial processes (Halassa, Fellin et al. 2007, Gerstner, Vanderheyden et al. 2012). As microglia and astrocytes can actively engage in synaptic function (Halassa, Fellin et al. 2007) and have been associated with alcoholism (Ponomarev, Wang et al. 2012), the SN preparation may be useful in investigating alcohol's effects on the neuroimmune system. Because the cell-type specificity in our SN preparation is not known, we used a comprehensive bioinformatics approach similar to Ponomarev et al. (Ponomarev, Wang et al. 2012) (Ponomarev, Wang et al. 2012) (Ponomarev, Wang et al. 2012) (Ponomarev, Wang et al. 2012) (Ponomarev, Wang et al. 2012) (Ponomarev, Wang et al. 2012) (Ponomarev, Wang et al. 2012) (Ponomarev, Wang et al. 2012) (Ponomarev, Wang et al. 2012) (Ponomarev, Wang et al. 2012), including identifying mRNAs which are co-expressed with known

astro-glial markers and examining the astro-glia mRNAs altered by alcohol consumption (Ponomarev, Wang et al. 2012).

Extra-nuclear splicing has been discovered as a process involved in synaptic structure and function (Glanzer, Miyashiro et al. 2005), and this process may explain why nuclear mRNAs were found in the SN preparation. Alternatively, there could be some nuclear contamination. The estimate of nuclear contamination in our SN preparation is based on two main measurements: (1) DAPI (4',6-diamidino-2- phenylindole) staining of nuclear DNA, showing no detected staining in the SNs (Supplementary Figure S2)(Sosanya, Huang et al. 2013). (2) Neun (a nuclear protein) western blots, showing the SN preparation decreases 75% of the Neun found in the TH (Sosanya, Huang et al. 2013). Although there is evidence for synaptic translation in postsynaptic neuronal compartments (Zalfa, Eleuteri et al. 2007), translation might also take place in the presynaptic compartment (axonal terminal) (Akins, Berk-Rauch et al. 2009). The SN preparation enriches for both the presynaptic and the postsynaptic fractions, and further research is warranted to determine whether alcohol differentially affects these two compartments.

In summary, we identified coordinated changes in mRNA expression in SNs and THs following chronic alcohol consumption. The expression changes in SNs from mouse amygdala corroborate that seen in the amygdala of human alcoholics and include overlapping changes in GABA, glutamate and neuroimmune pathways. Our results demonstrate that (1) the mouse chronic-drinking paradigm used in this study is sufficient to produce the same expression changes previously seen in human alcoholics and (2) the parallel changes are evident for the SN but not TH mouse transcriptome. Our results highlight the advantage of the mouse SN preparation for identifying therapeutic gene targets for alcohol dependence that are relevant in humans and for studying synaptic plasticity under normal and disease conditions.

CHAPTER 3: SYNAPTIC MICRORNAS COORDINATELY REGULATE SYNAPTIC MRNAS: PERTURBATION BY CHRONIC ALCOHOL CONSUMPTION

The work presented in this chapter has been published in 2016 in Neuropsychopharmacology. Most D, Leiter C, Blednov YA, Harris RA, Mayfield RD. Synaptic microRNAs Coordinately Regulate Synaptic mRNAs: Perturbation by Chronic Alcohol Consumption. 41(2):538-48. PubMed PMID: 26105134. Supplementary material to this work can be found in the online version of the manuscript at http://www.nature.com/npp/journal/v41/n2/suppinfo/npp2015179s1.html.

Local translation of mRNAs in the synapse plays a major role in synaptic structure and function. Chronic alcohol use causes persistent changes in synaptic mRNA expression, possibly mediated by microRNAs localized in the synapse. We profiled the transcriptome of synaptoneurosomes (SN) obtained from the amygdala of mice that consumed 20% ethanol (alcohol) in a 30-day continuous two-bottle choice test to identify the microRNAs that target alcohol-induced mRNAs. SN are membrane vesicles containing pre- and post-synaptic compartments of neurons and astroglia and are a unique model for studying the synaptic transcriptome. We previously showed that chronic alcohol regulates mRNA expression in a coordinated manner. Here, we examine microRNAs and mRNAs from the same samples to define alcohol-responsive synaptic microRNAs and their predicted interactions with targeted mRNAs. The aim of the study was to identify the microRNA–mRNA synaptic interactions that are altered by alcohol. This was accomplished by comparing the effect of alcohol in SN and total homogenate preparations from the same samples. We used a combination of unbiased bioinformatic methods (differential expression, correlation, co-expression, microRNA-mRNA target prediction, co-targeting, and cell type-specific analyses) to identify key alcohol-sensitive microRNAs. Prediction analysis showed that a subset of alcohol-responsive microRNAs was predicted to target many alcohol-responsive mRNAs, providing a bidirectional analysis for identifying microRNA–mRNA interactions. We found microRNAs and mRNAs with overlapping patterns of expression that correlated with alcohol consumption. Cell type-specific analysis revealed that a significant number of alcohol-responsive mRNAs and microRNAs were unique to glutamate neurons and were predicted to target

each other. Chronic alcohol consumption appears to perturb the coordinated microRNA regulation of mRNAs in SN, a mechanism that may explain the aberrations in synaptic plasticity affecting the alcoholic brain.

Introduction

Local translation of synaptic mRNAs is essential for the functional properties of brain cells (Raab-Graham, Haddick et al. 2006, Wang, Kim et al. 2009). The extensive neuroadaptations associated with alcohol dependence are likely caused by persistent changes in the expression of hundreds of mRNAs (Mayfield, Lewohl et al. 2002, Ponomarev, Wang et al. 2012, Nunez, Truitt et al. 2013). Many of the alcohol-responsive adaptations are related to synaptic structure and function and may be caused by coordinated changes in local mRNA translation (Wang, Martin et al. 2010, Nunez and Mayfield 2012). MicroRNAs are short, noncoding RNAs that can regulate the translation of many target mRNAs, and this process is known to occur in the synaptic compartments of the cell (Lugli, Larson et al. 2005, Lugli, Torvik et al. 2008, Smalheiser and Lugli 2009, Sosanya, Huang et al. 2013). The ability of microRNAs to regulate mRNAs provides a localized regulatory system that may be important in the treatment of alcoholism.

Exposure to alcohol and other drugs of abuse modulates microRNA expression in the brain (Pietrzykowski, Friesen et al. 2008, Eipper-Mains, Kiraly et al. 2011, Lewohl, Nunez et al. 2011, Tapocik, Solomon et al. 2013). MicroRNAs have important roles in learning and memory (Konopka, Kiryk et al. 2010), and are also altered in addiction-related behaviors, such as cocaine conditioned place preference (Chandrasekar and Dreyer 2011), cocaine-seeking behavior (Novak, Halbout et al. 2010), and self-administration of alcohol (Tapocik, Solomon et al. 2013). Little is known about the microRNAs involved in the regulation of synaptic mRNA translation during alcohol dependence. Studies investigating the effects of chronic alcohol consumption in standard tissue (total homogenate, TH) preparations found a persistent change in the expression of microRNAs and their target mRNAs

in humans, mice, and rats (Lewohl, Nunez et al. 2011, Gorini, Nunez et al. 2013, Nunez, Truitt et al. 2013, Tapocik, Solomon et al. 2013). However, the standard TH preparation likely underestimates the number and magnitude of alcohol responsive transcripts localized in the synapse (Lewohl, Nunez et al. 2011, Most, Ferguson et al. 2015).

Synaptoneurosomes (SN) (Hollingsworth, McNeal et al. 1985, Quinlan, Philpot et al. 1999, Raab-Graham, Haddick et al. 2006, Sosanya, Huang et al. 2013) contain membrane vesicles of pre- and postsynaptic compartments of neurons as well as Peri-synaptic compartments of astrocytes and microglia and offer an improved model for studying the synaptic transcriptome. We recently showed that alcohol-induced mRNA changes are greater in SN compared with TH (Most, Ferguson et al. 2015). Here, we profiled the microRNA transcriptomes from paired SN and TH samples of mouse amygdala after a voluntary alcohol consumption paradigm. We identified alcohol-induced microRNAs that were correlated with alcohol consumption and also identified their predicted mRNA synaptic targets.

Materials and Methods

Animal Housing and Alcohol Consumption

Adult (two-month-old) C57BL/6J female mice were maintained at the University of Texas at Austin Animal Resources Center. Mice were group-housed and given a one-week acclimation period in combined housing and another week to acclimate to the bottle positions in individual housing. Food and water were provided ad libitum and monitored daily, as were the temperature and reverse light/dark cycle. Mice underwent a 30-day two-bottle choice paradigm with continuous (24-h) access to one bottle of 20% ethanol (referred to as alcohol) and one bottle of water (Blednov, Mayfield et al. 2012). A control group of mice received two bottles of water. Bottle weights were recorded daily, mice were weighed every four days, and the amount of alcohol consumed was calculated as g/kg/24h (see Supplementary Figure S1 for amounts of alcohol consumed). Alcohol bottle positions were alternated daily to control for position preferences. The stage of the menstrual cycle was not

determined. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin and adhere to NIH Guidelines for the ethical care and use of animals in research.

RNA Extraction

As described previously (Most, Ferguson et al. 2015), after 30 days of the two-bottle choice drinking, 8 alcohol-consuming and 12 control mice were killed by cervical dislocation and decapitated at the beginning of the light phase of the light/ dark cycle. Brains were removed and washed for 1 minute with 1ml of ice-cold homogenizing buffer (HB) containing 20mM HEPES, 1 mM EDTA (pH 7.4), 40 U/ml RNAseOut (Invitrogen, CA), phosphatase inhibitor cocktail 3 (Sigma, MO) and protease inhibitors 'Complete' (Roche, IN). Brains were then placed in a coronal Zivic mouse brain slicer with a 0.5mm resolution (Zivic Instruments, PA) and sliced in the following coordinates in order to isolate extended amygdala: coronal level 56-66 (Bregma (−0.18)-(−1.155)) and 66–80 (Bregma (−1.155)-(−2.55)). The extended amygdala was dissected, placed in ice-cold HB (250µl), and homogenized for 1 minute using a VWR homogenizer and pestle (VWR, PA). To minimize homogenate loss, pestles were washed with 50µl HB after use and the wash was collected and added to the sample. Ten percent of the homogenate (30µl) were snap frozen in liquid nitrogen and stored at − 80 °C for subsequent RNA TH analysis. Paired SN samples were isolated from the remaining 270µl of the homogenate. Homogenates were filtered through a 100-µm-pore filter (Millipore, MA) and subsequently through a 5-µm-pore filter (Millipore); filters were washed with HB before use for protection from RNAse. To maximize yield, the filters were washed with 50µl HB after use and the wash was collected and added to the homogenate. The homogenate was then centrifuged at 14,000g for 20 minutes at 4°C in order to pellet the cell fraction containing SN. The supernatant was removed and the pellet was snap frozen and stored at −80°C for SN RNA analysis. Microscopy was used to further characterize the SN preparation (Most, Ferguson et al. 2015). RNA was extracted from 20 paired SN and TH samples using

the Direct-Zol RNA extraction kit (Zymo, Japan) with small IC extraction columns according to the manufacturer's instructions. The RNA was quantified using a Nano-Drop1000 (Thermo Fisher Scientific Inc., IL) and assayed for quality using an Agilent 2100 Tape Station (Agilent Technologies, CA; Supplementary Figure S2A). The criteria for RNA quantity and quality were as follows: total amount of RNA>500ng, 280/260>1.7, and RIN>6.5. We measured RIN of five of the control samples using the Bio-analyzer Nano kit (Agilent). To ensure that samples with high RINs also included high quality and quantity of microRNAs, the same five control samples were subjected to a small RNA analysis using the Bio-analyzer Small kit (Agilent). Micro-RNAs comprised >10% of the small RNA population in the samples (Supplementary Figure S2B).

Microarray Hybridization, Data Quality Assessment, and Analysis

Total RNA was extracted from the TH and SN samples. The homogenates were divided into two parts (90% was used for SN and 10% for TH). The 20 samples from the SN and 20 from the TH (40 total) were hybridized to 40 microRNA microarrays. Previously, we used 40 mRNA microarrays from these same mice (Most, Ferguson et al. 2015). Four samples per mouse were hybridized (SN microRNA, SN mRNA, TH microRNA, and TH mRNA). The RNA samples were divided for mRNA (Most, Ferguson et al. 2015) and microRNA (this study) analyses. The RNA targeted for microRNA analysis was labeled with flash-tag biotin HSR (Affymetrix, CA) and hybridized to GeneChip microRNA 3.0 Arrays (Affymetrix) at the University of Texas Southwestern Medical Center microarray facility in Dallas. Affymetrix microarrays show a high correlation with results obtained from other platforms such as qPCR (Kolbert, Feddersen et al. 2013, Mestdagh, Hartmann et al. 2014). This microarray platform uses annotations from miRBase version 17 and contains 19,724 probes for mature micro-RNAs from 153 organisms. For this study, we focused on the 1,111 mature mouse microRNAs detected on the array. The mRNA and microRNA data discussed in this publication have been

deposited in NCBI's Gene Expression Omnibus (Edgar, Domrachev et al. 2002) and are accessible through GEO Series accession number GSE51730.

We analyzed the array data using R programming language and Bioconductor packages (http://www.bioconduc tor.org). Preprocessing (RMA, background correction, and quantile normalization) of the microRNA data was performed with the Bioconductor Oligo package. Quality measures were taken before and after preprocessing using the Array Quality Metrics package (Kauffmann, Gentleman et al. 2009, Kauffmann and Huber 2010) to generate the principal component analysis (PCA). For all further analyses, only the mouse mature microRNA data were used. The microarray expression data were then analyzed using the Bioconductor Limma package according to the author instructions (Smyth 2004). This analysis is the main approach currently used for studying thousands of genes from microarrays. We used differential expression analysis between paired SN and TH samples (paired/dependent t-test; N=20 per group). For comparison of SN and TH expression levels, fold changes were calculated as the ratio of SN to TH. Fold changes greater than 1 are referred to as 'SN-enriched' and fold changes less than 1 are referred to as 'SN-depleted'.

Independent t-tests were used to compare the alcohol (N=8) and control (N=12) groups to determine the effect of alcohol within SN and TH groups. These tests were performed in two separate analyses (one for each preparation). Equal sample sizes are not required for this analysis. For comparison of alcohol and control expression levels, fold changes were calculated as the ratio of alcohol to control expression levels. Fold changes greater than 1 are referred to as 'upregulated' and fold changes less than 1 are referred to as 'downregulated'. P-values<0.05 were considered significant for all analyses in the study.

For microRNA co-expression analyses, we used weighted gene correlation (co-expression) network analysis (WGCNA; Langfelder and Horvath, 2008). Individual mice can consume slightly different amounts of ethanol, and we were interested in the time period that represented a consistent amount of drinking and chose the average consumption per mouse from day 10 to day 30.

The alcohol consumption was correlated with the expression data using Pearson correlation (N=8). However, a WGCNA network was calculated using the 20 SN samples, including both alcohol and control samples. We then determined which of the microRNA modules correlated with alcohol consumption. The WGCNA parameters were as follows for the SN: power 12, signed network, cutHeight 0.995, minModsize 60. For cell type enrichment analysis, we used lists from CamkIIa+ glutamate neurons and Gad+, somatostatin+ and parvalbumin+ GABA neurons (He, Liu et al. 2012).

We combined the microRNA data (see Table 3.1 for experimental design) with our previously published mRNA results (Most, Ferguson et al. 2015), which were obtained from the same samples.

Alcohol consuming mice N=8	SN	N=8 X 2=16
	TH	
Control mice N=12	SN	N=12 X 2=24
	TH	

TABLE 3.1: EXPERIMENTAL DESIGN OF THE STUDY

20 mice were allocated to either the alcohol or control group. Each mouse contributed an amygdala sample that was split into two parts, one for SN and one for TH. Total RNA was extracted from each of these preparations and was hybridized to microRNA microarrays. In our previous study (Most et al. 2015) we had hybridized the same samples to mRNA microarrays. Here, the total number of samples was 40: 8 SN and 8 TH, for a total of 16 alcohol samples; 12 SN and 12 TH, for a total of 24 control samples. Samples were individually hybridized to microarrays.

To determine alcohol-sensitive SN and TH modules, we used the 'alcohol-responsive mRNA' lists from Most et al. 2015 (Most, Ferguson et al. 2015). All P-values from the bioinformatics analyses were adjusted using the Benjamini–Hochberg method. For cell types and immune response enrichment, we used the following lists of genes: neurons, astrocytes and oligodendrocytes (Cahoy, Emery et al. 2008), microglia (cured from Oldham et al. 2008 (Oldham, Konopka et al. 2008)), and

glutamate/GABA (Sugino, Hempel et al. 2006). For cross species comparison, we used lists from alcohol vapor-treated rats (Tapocik, Solomon et al. 2013) and human alcoholics (Lewohl, Nunez et al. 2011) to identify conserved alcohol-responsive microRNAs. Drugs that potentially target the alcohol-responsive mRNAs and the 'mRNA targets of the alcohol-responsive microRNAs' were found using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Qiagen, CA).

Classification of microRNA–mRNA Interactions

Mouse target predictions for microRNAs and mRNAs were extracted from microRNA.org (version 8-2010). The micro-RNA.org resource comprises predictions computed by the miRanda algorithm (John, Enright et al. 2004, Betel, Koppal et al. 2010). The algorithm predicts microRNAs according to the number of putative target sites and the sum of alignment scores determined by both seed match type and seed match context. The predictions that were considered were those annotated as 'conserved microRNA' and 'good mirSVR score'. The individual binding locations can be found in http://www.microRNA.org. We then overlapped the list of mRNAs that were predicted to be targeted by alcohol-responsive micro-RNAs with the list of microRNAs that were predicted to target the alcohol-responsive mRNAs. Prediction analysis showed that a subset of alcohol-responsive microRNAs were predicted to target certain alcohol-responsive mRNAs and vice versa, providing a bidirectional analysis for identifying microRNA–mRNA interactions using a novel approach.

Quantitative Real-Time PCR (qPCR)

qPCR was used to validate the array data of microRNA expression levels in the same SN samples. First, RNA was DNase treated using the DNA-free Kit (Ambion, TX) and reverse transcribed into cDNA using the iScript Select cDNA Synthesis Kit (Bio-Rad, CA). Samples were then evaluated for the presence of genomic DNA by comparing GAPDH Cq values from RT+ and RT− reactions, using single threshold Cq determination. Then, RNA (4.5 ng) from four of the SN alcohol samples and five of the SN control samples was used to synthesize cDNA, using the TaqMan MicroRNA Reverse

Transcription Kit and the TaqMan PreAmp Master Mix, according to the manufacturer's protocol for creating custom RT and preamplification pools (Applied Biosystems, NY). qPCR was performed in triplicates using TaqMan Universal PCR Master Mix (no AmpErase UNG). FAM-labeled TaqMan MicroRNA Assays (Applied Biosystems) were used to amplify mmu-miR-137 (Assay ID 001129) and hsa-miR-9* (Assay ID002231). Normalized relative expression was determined with respect to the most stably expressed small nucleolar RNAs, Sno-234 (Assay ID 001234) and Sno-202 (Assay ID 001232), as determined by geNorm analysis (Vandesompele, De Preter et al. 2002). qPCR results were imported into qBase+ software, version 2.5 (Biogazelle, BE), where the ΔΔCt method was used (Hellemans, Mortier et al. 2007). Statistical analysis was completed in GraphPad Prism software, version 6.

Results

SN and TH microRNA Transcriptomes are Different in Control and Alcohol Samples

We studied the microRNA transcriptomes in paired SN and TH preparations to compare synaptic vs total cell microRNA expression. PCA revealed a distinct clustering of microRNA expression in the two preparations, while showing a homogenous sample population (no outliers detected) within each preparation (Figure 3.1A). The clustering was evident along the first principal component, indicating the largest variation stems from the distinct expression profiles of the preparations. A comparison of the individual microRNA expression levels on the arrays showed similar values in the SN and TH preparations as well as microRNAs enriched in SN ('SN-enriched') and microRNAs depleted in SN ('SN-depleted'; Figure 3.1B). We compared the mature mouse microRNA expression levels in SN and TH (non-treated control) and found 180 differentially expressed microRNAs. Eighty-one microRNAs were 'SN-enriched' showing up to an eightfold change in expression, and ninety-nine were 'SN-depleted' (Supplementary Table S2).

We then compared SN and TH samples (referred to as SN-alcohol and TH-alcohol) from mice that chronically consumed 20% ethanol in a two-bottle choice paradigm and found 153 differentially expressed microRNAs, 96 of which were enriched in SN (Supplementary Table S2). It is possible that alcohol exposure changes the expression or trafficking of microRNAs to the synapse, resulting in different SN-enriched microRNAs in alcohol samples compared with control. We assessed the overlap between the differentially expressed microRNAs in SN and TH under alcohol and control conditions and found 63 overlapping microRNAs present in both conditions (Figure 3.1C).

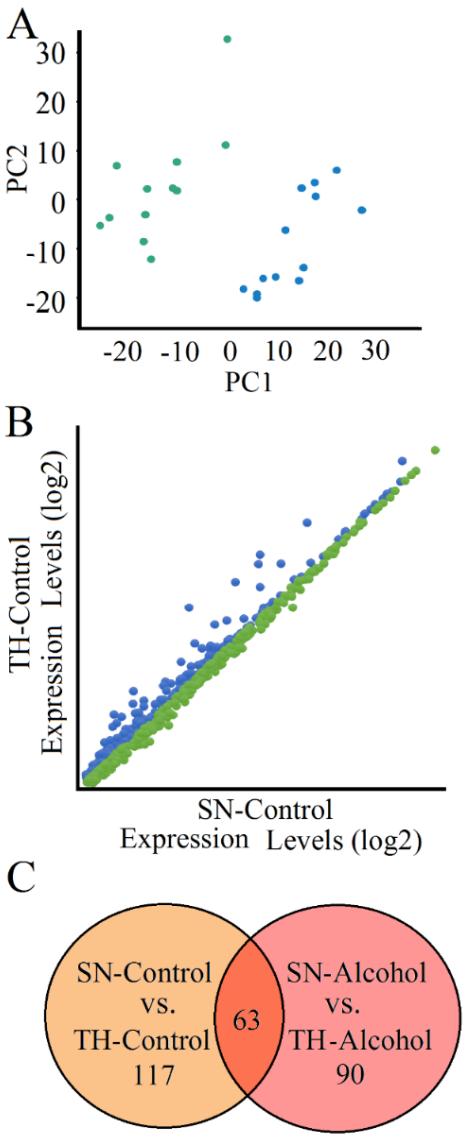


FIGURE 3.1: DIFFERENCES IN EXPRESSION PROFILES AS DETECTED IN SN AND TH PREPARATIONS

A. Principal component analysis of paired SN (green) and TH samples (blue). All microRNAs on the array (including those from different species) were used for this analysis (25,119 probes). The primary purpose for this analysis is quality control to show the overall detection of transcripts on the array and to facilitate comparison of the preparations. Only the mouse mature microRNAs will be discussed and presented in subsequent tables and figures. B. Expression levels of microRNAs in SN and TH preparations (control group only). For comparison of SN and TH expression levels, fold changes were calculated as the ratio of SN to TH. MicroRNAs below the diagonal are enriched in SN relative to TH and have a fold change greater than 1 (referred to as 'SN-enriched'; shown in green). MicroRNAs above the diagonal are depleted in the SN relative to the TH and have a fold-change less than 1 (referred to as 'SN-depleted'; shown in blue). C. Venn diagram showing the number of differentially expressed microRNAs from the SN-control/TH-control analysis and the SN-alcohol/TH-alcohol analysis, and the overlap between them. P-values <0.05 were considered significant.

Alcohol Consumption Alters microRNA Expression in SN and TH

We investigated alcohol's effects on SN microRNA expression and identified 65 mature mouse microRNAs that were differentially expressed between the alcohol and control samples (Table 3.2; Supplementary Table S2). Seventy-seven alcohol-sensitive microRNAs were differentially expressed in TH samples (Supplementary Table S2). Twenty microRNAs with the greatest fold changes are shown for SN and TH in Table 3.3.

MicroRNA type	Number of microRNAs investigated	Number of alcohol-responsive in SN	Number of alcohol-responsive in TH	Overlap between SN and TH
All species	25,119	1,377	1,623	77
Mouse mature	1,111	65	77	1

TABLE 3.2: ALCOHOL-RESPONSIVE MICRORNAS IN SN AND TH

Number of microRNAs (mature and premature/precursor) from all species are shown next to the mouse-specific microRNAs examined for this study. The total number of microRNAs in each group is shown next to the number of alcohol-responsive (P-values<0.05) microRNAs from that group, as detected in SN and TH preparations.

Alcohol-responsive microRNAs in SN	SN fold-change	SN P-value	TH fold-change	TH P-value	Alcohol-responsive microRNAs in TH	TH fold-change	TH P-value	SN fold-change	SN P-value
miR-1893	1.62	2.71E-02	0.91	6.36E-01	miR-1965	1.63	1.44E-02	1.06	7.66E-01
miR-875-3p	1.61	4.45E-03	0.78	9.24E-02	miR-207	1.61	1.98E-04	0.99	9.48E-01
miR-187*	1.56	2.87E-02	1.44	1.67E-01	miR-467d*	1.57	1.38E-02	0.68	1.04E-01
miR-187	1.51	1.68E-02	1.33	1.76E-01	miR-193*	1.53	1.44E-02	1.27	2.42E-01
miR-92a-2*	1.47	4.53E-02	1.18	4.59E-01	miR-3113*	1.45	1.98E-04	0.84	2.07E-01
miR-466n-3p	1.43	3.83E-04	0.87	2.84E-01	miR-574-5p	1.39	1.61E-02	0.63	8.91E-02
miR-669d-2*	1.38	1.67E-02	1.06	4.32E-01	miR-200c	1.35	2.95E-02	1.28	8.78E-02
miR-216b	1.38	8.53E-03	0.98	8.67E-01	let-7b*	1.32	2.11E-02	0.78	9.23E-02
miR-501-5p	1.37	4.41E-02	0.80	1.09E-01	miR-322	1.30	3.78E-02	0.99	9.32E-01
miR-5115	1.34	3.51E-02	0.79	2.15E-01	miR-30c-2*	1.28	2.79E-02	1.03	8.43E-01
miR-18a	0.65	1.41E-02	1.00	9.98E-01	miR-9	0.63	1.93E-02	1.18	3.64E-01
miR-377*	0.65	1.73E-02	1.13	4.23E-01	miR-539-5p	0.62	1.40E-03	0.90	5.57E-01
miR-466g	0.65	4.11E-02	1.33	1.97E-01	miR-411*	0.62	7.47E-03	0.83	1.73E-01
miR-135a-2*	0.64	4.82E-03	0.74	2.09E-01	miR-3068	0.61	2.32E-02	0.95	8.25E-01
miR-466f	0.64	3.33E-02	0.85	3.12E-01	miR-3473	0.60	1.67E-02	1.03	8.39E-01
miR-5099	0.63	4.18E-02	0.90	3.74E-01	miR-5100	0.60	5.64E-04	1.28	9.73E-02
miR-344c	0.59	8.78E-04	0.83	2.63E-01	miR-5097	0.58	1.07E-02	1.23	2.25E-01
miR-34c*	0.58	3.78E-02	0.77	2.98E-01	miR-132*	0.54	9.32E-03	0.96	6.99E-01
miR-1187	0.58	2.09E-03	1.48	1.75E-01	miR-720	0.52	3.52E-03	1.20	3.46E-01
miR-466f-3p	0.43	3.83E-04	1.59	1.37E-01	miR-1298	0.37	1.78E-02	0.56	8.19E-02

TABLE 3.3: ALCOHOL-RESPONSIVE MICRORNAS WITH THE LARGEST FOLD-CHANGE IN SN (LEFT) AND TH (RIGHT)

The top 10 most upregulated microRNAs (top rows) and most downregulated microRNAs (bottom rows) in SN (left) and TH (right). Left side of the table contains the fold changes and P-values as detected in SN, whereas the right side of the table contains the fold changes and P-values detected in TH. For comparison of alcohol and control expression levels, fold changes were calculated as the ratio of alcohol to control expression levels. Fold changes greater than 1 are referred to as 'upregulated' and fold changes less than 1 are referred to as 'downregulated'. P-values<0.05 were considered significant. For example, miR-1893 is upregulated in SN, but is downregulated in TH following alcohol treatment.

There were 23 and 39 upregulated microRNAs with average fold-change magnitudes of 33% and 26% in SN and TH, respectively (Figure 3.2A). There were 42 and 38 downregulated microRNAs with average fold-change magnitudes of 25% and 27% in SN and TH, respectively. The average fold changes between the preparations were not statistically significant (Supplementary Table S3). The fold changes in SN and TH were not influenced by inherent bias associated with either preparation, as shown in a volcano plot of the fold changes and P-values for SN and TH (Figure 3.2B).

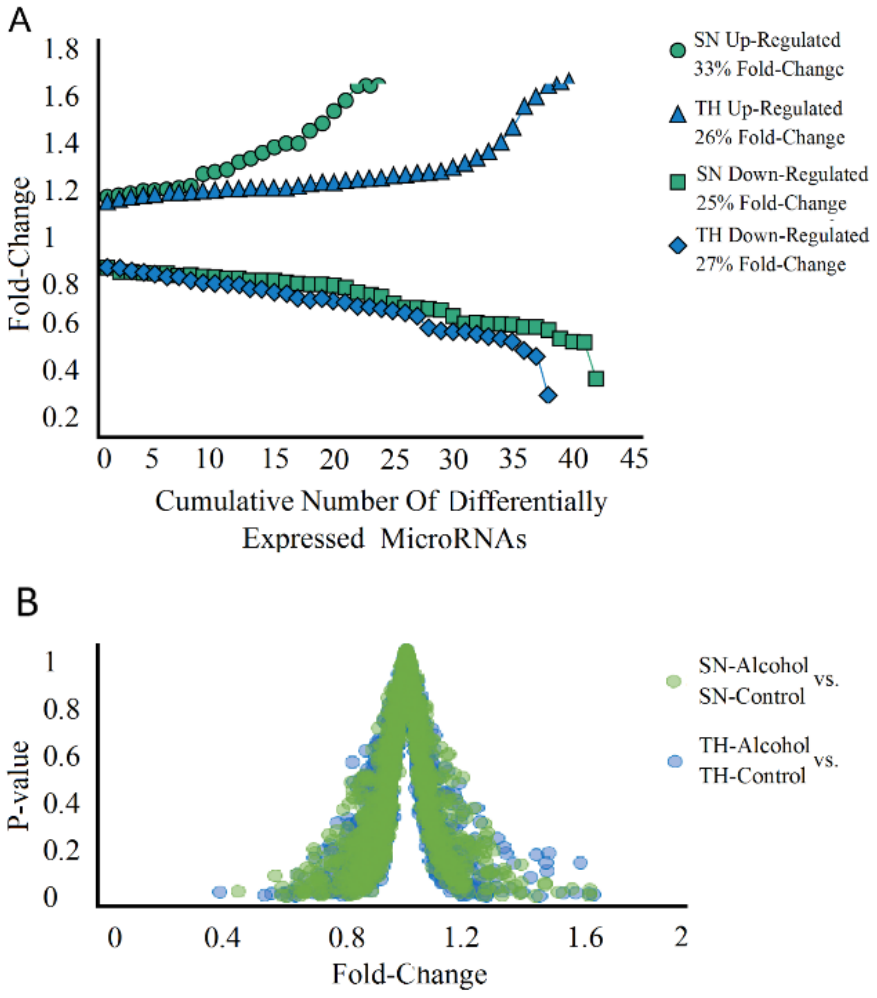


FIGURE 3.2: ALCOHOL-INDUCED MICRORNAS ARE DIFFERENT IN SN AND TH

A. Number of alcohol-responsive microRNAs in SN and TH. Alcohol-induced microRNA expression fold-changes are shown on the y-axis. For comparison of alcohol and control expression levels in each of the preparations, fold changes were calculated as the ratio of alcohol to control expression levels (SN-alcohol/SN-control and TH-alcohol/TH-control). Fold changes greater than 1 are referred to as 'upregulated' and fold-changes less than 1 are referred to as 'downregulated'. B. Volcano plot (scatter plot) of fold changes and P-values of the effects of alcohol on microRNAs in SN and TH.

Two microRNAs were chosen for qPCR validation: miR-137, which was found to be alcohol-responsive in SN, and miR-9*, which was not alcohol responsive in SN. Pearson correlation for expression levels between the qPCR and arrays for miR-137 showed r=0.69. A one-tailed Students' t-

test was used to test the significance of the correlation between the array and qPCR data. The correlation for miR-137 was significant (P=0.021). Pearson correlation for expression levels between qPCR and microarrays for miR-9* showed r=0.70 and this was also significant (P=0.018).

Synaptic microRNAs Coordinately Regulate Synaptic mRNAs Following Alcohol Consumption

Co-expressed microRNAs may regulate their mRNA targets in response to alcohol treatment. We used WGCNA to create a co-expression network to group microRNAs with similar patterns of expression into modules. We then identified the modules that were significantly correlated with alcohol consumption and found 610 microRNAs that were co-expressed within six different modules (Supplementary Table S2). Sixty-five microRNAs were differentially expressed in SN (Table 3.2), and 35 were co-expressed within these modules (Figure 3.3A). We identified microRNAs that correlated with alcohol consumption and found 74 in SN (Supplementary Table S2; average correlation: r>0.78), 48 of which were co-expressed within the six microRNA modules.

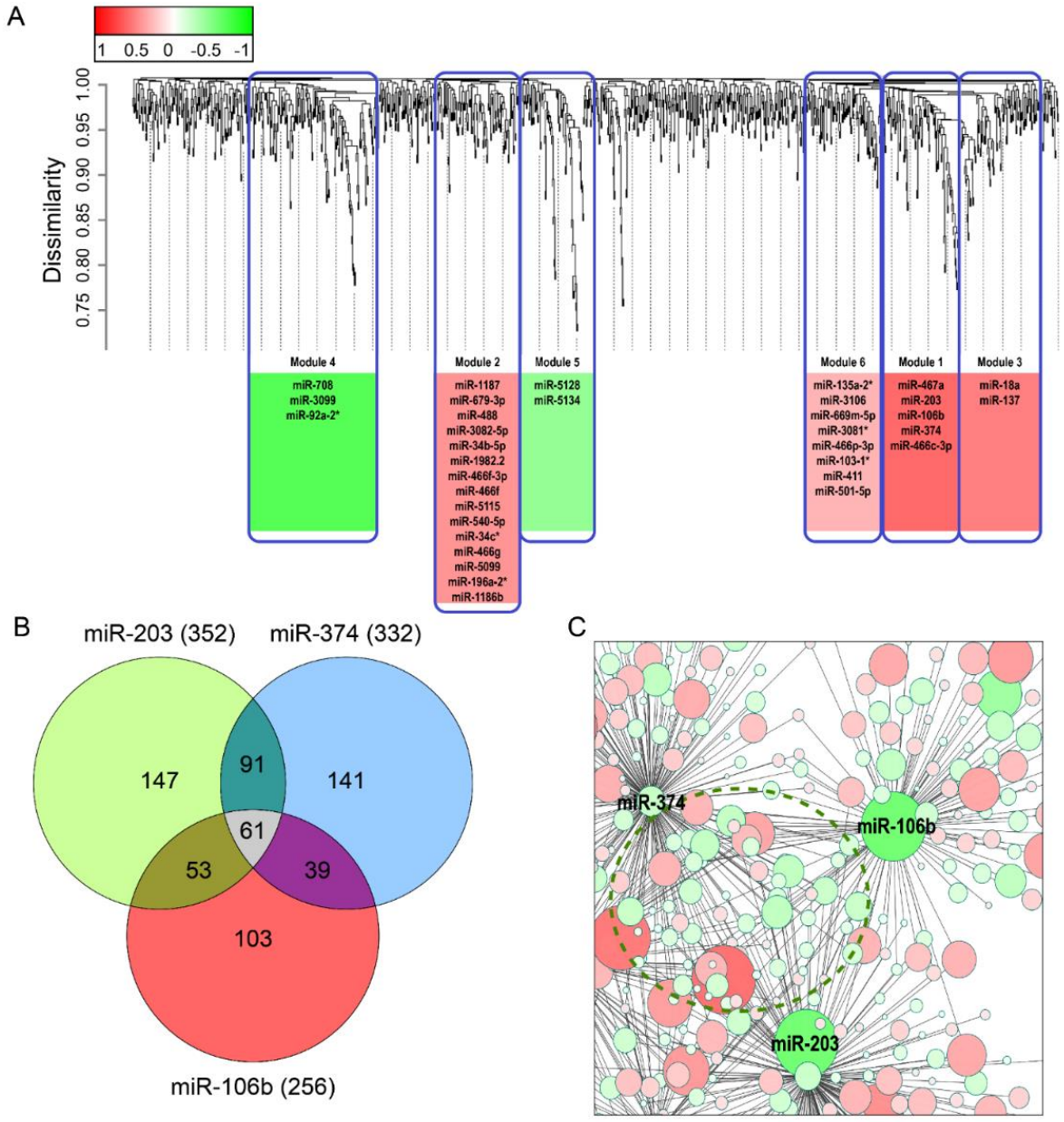


FIGURE 3.3: ALCOHOL INDUCES COORDINATED EXPRESSION OF MICRORNAS THAT ARE CORRELATED WITH ALCOHOL CONSUMPTION

A. Hierarchical clustering of microRNAs from SN, including both alcohol and control data. The microRNAs are arranged by covariance similarity; thus, microRNAs under the same branch have greater expression similarity than those outside the branch. The dissimilarity among microRNAs is represented in the y-axis. The six different modules are shown in boxes. The width of the box represents the number of microRNAs co-expressed in that module. The correlation of each module with alcohol consumption is shown as a heat map (red represents positive correlation with consumption and green represents negative). The microRNA list contains co-expressed

microRNAs which are also alcohol-responsive. The microRNAs in the gap between the modules are ones that did not pass the co-expression threshold as defined by the WGCNA and were not included in any module. B. Examples of three co-expressed microRNAs and the number of overlapping predicted alcohol-responsive mRNAs. C. Examples of microRNA predicted interactions. The greater the color intensity, the greater the fold-change magnitude (red is upregulated and green is downregulated). The unmarked circles represent mRNAs. The dotted circle emphasizes mRNAs that are co-targeted by the illustrated microRNAs.

We next utilized mRNA expression data (alcohol-responsive modules of co-expressed mRNAs) from our previous study (Most, Ferguson et al. 2015) to identify the alcohol-responsive microRNAs co-expressed with the mRNA modules. The modules were defined as astrocytic, microglial, or neuronal if they contained a significant number of the cell type associated mRNAs based on a hypergeometric distribution. The astrocyte modules overlapped with the microglia ones and were therefore combined. Cell type-specific analysis revealed 16 microRNAs that were significantly correlated with the alcohol-responsive mRNA modules (Figure 3.4).

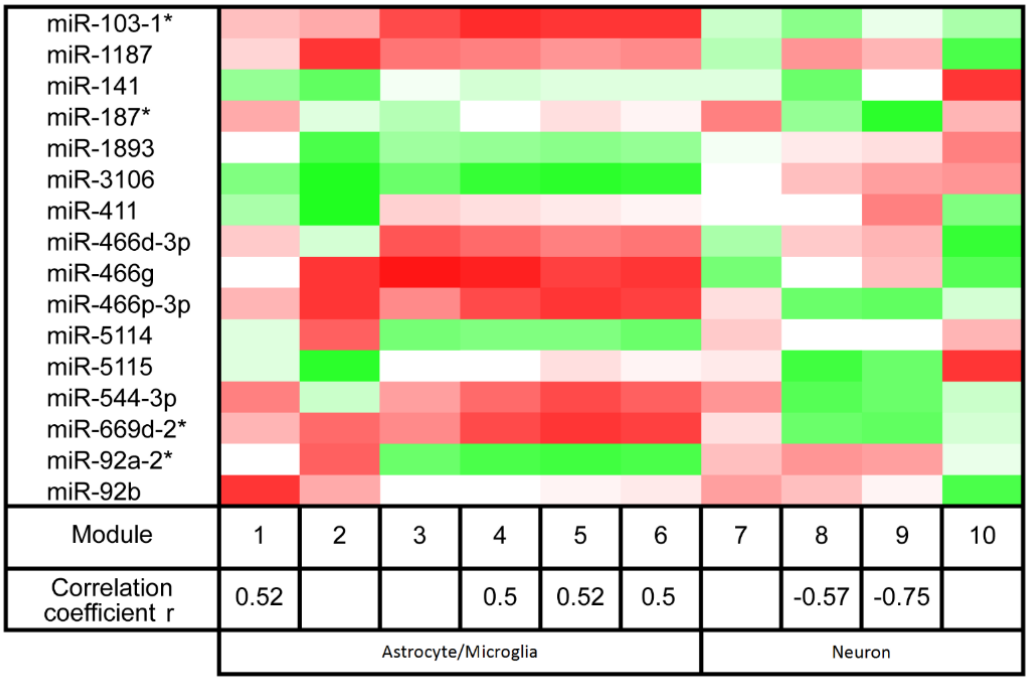


FIGURE 3.4: MICRORNA–MRNA INTERACTIONS ARE COORDINATELY EXPRESSED IN RESPONSE TO ALCOHOL AND ARE ASSOCIATED WITH SPECIFIC CELL TYPES

Shown are the alcohol-responsive mRNA modules and their correlation to individual microRNAs found in SN. The 10 alcohol-responsive mRNA modules are shown. The modules' correlation with consumption for the six alcohol-responsive modules is shown as

r-values (Pearson's correlation coefficient). Alcohol-responsive mRNA module correlation to individual microRNAs is represented as a heat map, with red representing a positive correlation and green representing a negative. Cell type mRNA enrichment is also shown. Modules 1–6 were enriched with astrocytic/microglial mRNAs, whereas modules 7–10 were enriched with neuronal mRNAs. The 16 microRNAs that were significantly correlated with at least one mRNA module are shown (mRNA data are from Most et al. 2015).

Synaptic microRNA–mRNA Interactions are Regulated by Alcohol

MicroRNA–mRNA interactions were constructed using target predictions from the 2010 miRanda database for 'good mirSVR scores' and 'conserved microRNAs' (http://www.microrna.org). From these predicted interactions, the alcohol-responsive microRNAs were predicted to target 1,039 target mRNAs of the 1,531 that were identified as alcohol-sensitive in Most et al. 2015 (Most, Ferguson et al. 2015). The alcohol-responsive mRNAs were predicted to be targeted by 15 of the 65 alcohol-responsive microRNAs (Table 3.4). The 15 micro-RNAs showed 15–51% change. We found that 250 of the mRNAs were predicted to be targeted by more than five of the alcohol-responsive microRNAs (Supplementary Table S4).

Number of alcohol-responsive RNAs	Number of predicted targets	Overlap between alcohol-responsive and predicted tragets
1,531 mRNA	13,857 mRNAs	1,039 mRNAs
65 microRNAs	238 microRNAs	15 microRNAs

TABLE 3.4: NUMBER OF ALCOHOL-RESPONSIVE MRNAS, MICRORNAS AND THEIR PREDICTED INTERACTIONS IN SN

The number of alcohol-responsive microRNAs and mRNAs is shown together with their predicted targets, as defined by the miRanda mouse database for interactions. The overlapping participants between the alcohol-responsive microRNAs/mRNAs and the predicted targets of each are shown, and were termed 'bidirectional' interactions. The data for the alcohol-responsive mRNAs were taken from Most et al. 2015. P-values<0.05 were considered significant.

We identified mRNAs cooperatively targeted by co-expressed microRNAs by combining the mRNA predicted target analysis with the microRNA co-expression data (Table 3.5).

microRNA	microRNA fold-change	microRNA P-value	Number of mRNA targets	Sum of scores	Co-expression	Overlapping targets for the co-expressed microRNAs
miR-203	0.66	1.63E-02	393	-157.76	Module 1	6330408A02Rik, Aebp2, Ank2, Ankfy1, Apc Asph, Camta1, Cdh8, Cobll1, Crbn, Dclk2, Dph3, Dpp3, Eif4a2, Etl4, Fam135a, Foxj3, Gria2, Hace1, Hnrnpa2b1, Hsd17b11, Huwe1, Ivns1abp, Maf, Mbd3l2, Mbd5, Mef2c, Mll3, Mtdh, Myt1l, Napepld, Narg2, Ndrg3, Necap1, Neto2, Oat, Ocrl, Osbp2, Pbrm1, Pbx1, Pja2, R3hdm1, Rgs7bp, Runx1t1, Scn1a, Sgk3 Ski, Slc30a1, Slc39a10, Srpk2, Syap1, Syncrip, Tmem209, Tmem87b, Trim37, Tshz3, Ube3a, Unc80, Zc3h6, Zfp644, Zxda.
miR-374	0.85	2.90E-02	366	-181.00		
miR-106b	0.83	3.43E-02	282	-129.25		
miR-488	0.83	1.20E-02	290	-123.16	Module 2	1600014C10Rik, 4833424O15Rik, AI314180, Acot11, Ahcyl1, Ankfy1, Ap2b1, Atl2, Camta1, Capn6, Car10, Cdc37, Cdc37l1, Cinp, Crbn, Cyld, Dgcr6, Etl4, Fam126b, Fbxo3, Gabrb2, Glce, Grm7, Gtf3c2, Hps5, Kif1b, Med18, Mllt3, Msi2, Mtdh, Myt1l, Ndst1, Pcdh10, Pja2, Pkp4, Prkd1, R3hdm1, Rab21, Rapgef4, Rarb, Rbbp9, Rftn2, Rnf4, Sec16a, Sesn1, Slc22a17, Slc35e3, Smc6, Snx12, Spnb2, Stx8, Tbxas1, Tcf25, Tle1, Tmem209, Tprkb, Tshz1, Tshz3, Tspan7, Unc80, Usp13, Utp6, Vps41, Vwa5b2, Zc3h14, Zfp644.
miR-34b-5p	0.66	1.48E-02	246	-84.36		
miR-137	0.65	2.03E-02	243	-133.62	Module 3	2700060E02Rik, 4732418C07Rik, Aebp2, Ahcyl1, Ank2, Ank3, Asph, Atl2, Cdk13, Crtc3, Ctdspl, Dgcr6, Dut, E2f6, Fry, Gatad1 , Gigyf1, Hnrpdl, Kdm5b, Khdrbs3, Lingo2, Lrrc16a, Maf, Mbnl2, Mfsd6, Mll1, Msi2, Nme7, Nrg1, Osbp2, Ppp2r5c, Rab8a, Rin2, Sae1, Scamp2, Scn1a, Seh1l, Slc13a3, Smc6, Sntg2, Snurf, Spag9, Stx8, Syncrip, Tmem87b, Ybx1, Zfp804a.
miR-18a	0.65	1.35E-02	198	-75.92		
miR-708	0.84	1.51E-02	192	-70.24	Module 4	Asph, Azi2, E2f6, Foxj3, Gpt2, Gucy1a3, Hspa9, Lipa, Mia3, Mtpap, Ocrl, Palld, Phf17, Pja2, Prkar1a, Pros1, Rapgef6, Rfx1, Robo2, Seh1l, Sgms1, Socs5, Txnip, Unc5c, Ybx1, Zfp804a.
miR-92b	1.19	4.40E-02	190	-90.99		
miR-365	1.18	4.45E-02	156	-58.25	Module 5	
miR-411	0.84	3.89E-02	153	-66.06	Module 6	
miR-141	0.87	4.10E-02	338	-144.24	NA	
miR-216b	1.35	1.49E-02	230	-101.41		
miR-92a	1.19	3.62E-02	201	-94.12		
miR-187	1.50	1.85E-02	64	-22.28		

TABLE 3.5: CO-EXPRESSED ALCOHOL-RESPONSIVE MICRORNAS AND THEIR OVERLAPPING MRNA TARGETS

Fifteen alcohol-responsive microRNAs and the number of predicted alcohol-responsive mRNAs (as identified in Most et al. 2015) is shown. The predictions were calculated using the miRanda database. The sum of each of the mirSVR scores per microRNA is shown for all predicted interactions per microRNA, with the scores representing the relative probability of occurrence of the microRNA–mRNA interactions. The WGCNA co-expression module for each of the microRNAs and the number of overlapping predicted mRNA

targets for the microRNAs in the same module are shown. “NA” represents alcohol-responsive microRNAs which were not co-expressed with other microRNAs, and therefore do not have overlapping mRNA targets with other microRNAs. For comparison of alcohol and control expression levels, fold changes were calculated as the ratio of alcohol to control expression levels. Fold changes greater than 1 are referred to as ‘upregulated’ and fold changes less than 1 are referred to as ‘downregulated’. $P<0.05$ were considered significant.

MiR-106b, miR-203, and miR-374 are examples of co-expressed microRNAs that were predicted to target 61 overlapping targets (Figure 3.3B). MicroRNAs can be negatively or positively correlated with their mRNA targets; e.g., miR-106b, miR-203, and miR-374 were all downregulated and were predicted to target both up- and downregulated mRNAs (Figure 3.3C).

The relationship among the alcohol-responsive micro-RNAs and the cell type specific glutamate and GABA microRNAs was examined using microRNA records (He, Liu et al. 2012). We identified 10 differentially expressed microRNAs that were unique to CamkIIa+ glutamate neurons, 5 unique to Gad+ GABA neurons, 9 unique to somatostatin+ GABA neurons but none unique to parvalbumin+GABA neurons. In addition, 9 of the 15 alcohol sensitive microRNAs were previously shown to be specific to glutamate neurons, and 8 of the 9 were highly predicted to target the alcohol-responsive glutamate mRNAs (Gria2, Grina, Grm7, and Grip1; Table 3.6).

microRNA	Grm7	Gria2	Grina	Grip1
miR-106b	-0.58	-1.03	NA	NA
miR-137	-0.69	NA	NA	NA
miR-18a	NA	-0.23	-0.34	-0.25
miR-203	NA	-0.52	NA	-0.39
miR-374	-0.77	-0.27	NA	NA
miR-411	NA	-0.68	NA	NA
miR-708	NA	NA	NA	NA
miR-92a	-0.70	NA	NA	NA
miR-92b	-0.70	NA	NA	NA

TABLE 3.6: ALCOHOL-RESPONSIVE MICRORNAS AND THEIR MRNA TARGETS THAT ARE SPECIFIC TO GLUTAMATE NEURONS

Nine glutamate-specific microRNAs of the 15 predicted alcohol-responsive microRNAs are shown. The numbers in the table represent the mirSVR scores for the predicted interactions between a microRNA and its glutamate-specific predicted mRNA targets (as defined by the miRanda mouse database for interactions). "NA" means no interactions were predicted for that specific microRNA with a glutamate mRNA. Alcohol-responsive mRNA data were taken from Most et al. 2015. Grm7 (glutamate receptor, metabotropic 7), Gria2 (glutamate receptor, ionotropic, AMPA2, also known as GluA2), Grina (glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1, glutamate binding), and Grip1 (glutamate receptor-interacting protein 1).

Discussion

The compartmentalization of RNA in cells allows for rapid responses to stimuli and may be important for the neuroadaptations in response to chronic alcohol consumption. The aim of this study was to identify the synaptic microRNAs that are altered by alcohol consumption and to propose microRNA–mRNA synaptic interactions that maybe changed by chronic alcohol. We compared the effect of alcohol in paired SN vs TH samples and identified those changes that were specific to the SN. Our data indicate that the microRNAs in the SN and TH respond differently to alcohol exposure: there was only one common alcohol responsive microRNA, miR-411, between the preparations (Supplementary Table S3). Such a small overlap was unexpected and underscores the advantage of the SN in examining discrete, localized responses to alcohol. We identified microRNAs that were SN enriched in alcohol but not control samples and vice versa. If only the magnitude of treatment fold changes for the same microRNA was larger in SN compared with TH, this might suggest that synaptic

enrichment was responsible for the differences. However, changes in magnitude do not explain all of the differences that we observed which appear to be both qualitative (different microRNAs) and quantitative (different amounts of the same microRNA). The differences between SN and TH are likely due to localized effects of alcohol and it is possible that alcohol changes the trafficking of microRNAs to the synapse, resulting in unique SN-enriched microRNAs.

This regimen of alcohol consumption causes extensive and coordinated changes in gene expression in the brain, suggesting a network regulator such as a microRNA may be involved (Lewohl, Nunez et al. 2011). The question remains regarding how, or if, alcohol affects synaptic pathways through synaptic microRNA regulation.

This is the first study to use SN profiling of microRNA and mRNA obtained from the same samples, enabling detection of alcohol-responsive synaptic microRNAs and mRNAs and the predicted interactions between them. We used a combination of unbiased methods to reveal key microRNAs and their targets. Chronic alcohol consumption caused robust changes in synaptic microRNA expression levels consistent with those seen in human alcoholics (Lewohl, Nunez et al. 2011) and in other animal models of dependence (Gorini, Nunez et al. 2013, Nunez, Truitt et al. 2013, Tapocik, Solomon et al. 2013). We further identified microRNAs with overlapping patterns of expression that correlated with alcohol consumption.

Previous studies used different experimental conditions, such as alcohol paradigms, species, gender, and brain regions, and we were interested in identifying potential conserved microRNAs that extend across all these different conditions. We suggest that a conserved microRNA could potentially be important in human disease. We employed many different bioinformatic approaches, such as co-expression and co-targeting, to identify the overlapping microRNAs and found some evidence that these are conserved among different species and genders. Further studies will be needed to validate the individual interactions. Nevertheless, the combined approaches provide a list of potential alcohol-sensitive interactions in the synapse that are candidates for further study.

We found the following conserved microRNAs in our study and that of an alcohol vapor exposure study in rats: miR-137, miR-187, miR-18a, miR-34c*, miR-369*, miR-374, miR-382*, miR-423, miR-488, and miR-92b (Tapocik, Solomon et al. 2013). Differentially expressed SN microRNAs in the current study also overlapped with differentially expressed microRNAs from human alcoholics (Lewohl, Nunez et al. 2011). These were miR-18a, miR-203, miR-369, miR-374, miR-92a, and miR-423.

We analyzed the differentially expressed mRNAs as potential targets for known drugs using IPA software and found three of the few drugs currently used to treat alcohol dependence (baclofen, disulfiram, and acamprosate) in the list. Using the differentially expressed microRNAs for the IPA analysis, we identified 26 drugs, 10 of which were also in the list of drugs identified using differentially expressed mRNAs. Seven of the ten drugs are FDA approved (Table 3.7), with several of these having links with alcohol actions For example, aminophylline blocks the behavioral effects of alcohol in mice (Soares, Patrocínio et al. 2009) and has antidepressant effects after alcohol exposure (Escudeiro, Soares et al. 2013).Theophylline blocks alcohol withdrawal-induced hyperalgesia (Gatch and Selvig 2002). Other drugs discovered from IPA were rasagiline (treatment for Parkinson's disease) and vorinostat, regorafenib, gemcitabine (paclitaxel), and romidepsin (treatments for cancer). The mechanisms of these drugs include histone deacetylase inhibition, adenosine receptor antagonism, phosphodiesterase inhibition, anti-inflammatory actions, and inhibition of monoamine oxidase and tyrosine kinase.

Drugs for alcohol targets	Molecular mechanisms	Potential uses
Belinostat Pyroxamide **Romidepsin** **Vorinostat**	Histone deacetylase inhibitor	Cancer
Gemcitabine/Paclitaxel	DNA polymerase inhibitor	
Tributyrin	Adenosine receptor antagonist	
Regorafenib	Tyrosine kinase receptor inhibitor	
Rasagiline	Monoamine oxidase inhibitor	Parkinson's disease
Aminophylline **Theophylline**	Phosphodiesterase inhibitor and adenosine receptor antagonist	Respiratory diseases

TABLE 3.7: DRUGS IDENTIFIED BY IPA THAT POTENTIALLY TARGET ALCOHOL-RESPONSIVE MRNAS

The alcohol-responsive mRNAs and the 'mRNA targets of the alcohol-responsive microRNAs' were overlapped to identify drugs known to affect these targets. Drug names, molecular mechanisms, and their current potential uses are shown. The data for the alcohol-responsive mRNAs were taken from Most et al. 2015. The seven drugs in bold are FDA approved.

We used a bidirectional approach to predict synaptic microRNA–mRNA interactions that were sensitive to alcohol (1,039 mRNAs and 15 microRNAs). Of these RNAs, 99 mRNAs and 9 microRNAs were unique to glutamate neurons. Notably, eight of these nine microRNAs were predicted to target alcohol-responsive mRNAs, such as CamkII, Gria2, Grina, Grm7, and Grip 1. Moreover, miR-203, miR-18a, and miR-374 were among the glutamate microRNAs that overlapped with the human data set (Lewohl, Nunez et al. 2011). These results suggest that alcohol regulates synaptic microRNAs, which in turn affect the expression of mRNAs in glutamate synapses and may partially explain the glutamate system dysregulation seen in alcoholics (Tsai and Coyle, 1998). We used the miRanda database to examine the most probable interactions that are regulated by alcohol consumption. This provides

further support for the predicted interactions, but it is important to note that these require direct validation to define their role in alcohol consumption.

A single microRNA has the potential to target many alcohol-responsive mRNAs (Nunez and Mayfield 2012, Tapocik, Solomon et al. 2013). This mechanism may be of particular importance in the synaptic proteome where slight adaptions can greatly impact synaptic plasticity. Alcohol-responsive microRNAs were significantly correlated with astrocytic, microglial, and neuronal modules. The co-expression of a microRNA with a network of alcohol-responsive mRNAs supports the role of microRNAs as master regulators in the synapse. The biological pathways associated with the mRNA modules include long-term potentiation and depression, glutamate and neuroimmune signaling, RNA processing, etc., suggesting the regulation of microRNAs in multiple processes. As for many other diseases (Maciotta, Meregalli et al. 2013), the neuroadaptations associated with alcohol dependence likely rely on many mRNAs. A subset of the mRNA changes may be driven by only a small number of microRNAs, each with the ability to target multiple mRNAs, thereby impacting alcohol-mediated responses and therapeutic strategies.

CHAPTER 4: SYNAPTIC MICRORNA-411 REDUCES ALCOHOL CONSUMPTION THROUGH HOMEOSTATIC INTERACTIONS WITH GLUA2

Introduction

Chronic drug and alcohol use causes widespread neuroadaptations in the brain, producing re-organization of synaptic structure and function (Nestler 2001, Kalivas and Volkow 2005, Kauer and Malenka 2007, Robison and Nestler 2011), which are likely the result of the abnormal expression of a plethora of genes in the brain (Mayfield, Lewohl et al. 2002, Eipper-Mains, Kiraly et al. 2011, Ponomarev, Wang et al. 2012, Gorini, Harris et al. 2014). An effective treatment for alcoholism therefore would likely require simultaneous regulation through the targeting of many of those genes. Given the complexity of the disease, it is not surprising that few pharmacotherapies have been identified for treating alcohol addiction and preventing relapse (Nutt, King et al. 2010, Organization 2014, Baingana, al'Absi et al. 2015).

MicroRNAs are small non-coding RNAs (Ambros 2001, Lee and Ambros 2001), that can control the translation of many genes, making them 'master regulators' of cellular gene expression (Miranda, Pietrzykowski et al. 2010). They are highly abundant in the brain and are also altered in response to chronic alcohol consumption in both humans and animal models. Alcohol-induced changes in microRNAs have been associated with development of cellular tolerance to alcohol (Pietrzykowski, Friesen et al. 2008), cellular reward mechanisms (Li, Li et al. 2013) regulation of alcohol consumption and preference (Bahi and Dreyer 2013, Li, Li et al. 2013, Tapocik, Barbier et al. 2014), episodes of binge drinking (Darcq, Warnault et al. 2014), withdrawal (Tapocik, Barbier et al. 2014) and alcohol-induced conditioned-place preference (Chandrasekar and Dreyer 2011, Bahi and Dreyer 2013). The extensive and coordinated alcohol-induced changes in gene expression may be driven by microRNA changes (Lewohl, Nunez et al. 2011, Mayfield and Nunez 2012, Mulligan, DuBose et al. 2013, Nunez, Truitt et al. 2013, Tapocik, Solomon et al. 2013, Mamdani, Williamson et al. 2015, Most, Leiter et al. 2016, Smith, Lopez et al. 2016), or alternatively, it could be that the mRNA changes drive the

microRNA changes (Nunez, Truitt et al. 2013). Nonetheless, the co-expression of microRNAs with a network of alcohol-responsive mRNAs supports the role of microRNAs as key regulators (Most, Leiter et al. 2016) (Gorini, Nunez et al. 2013).

Given that alcohol induces changes in synaptic structure and function, it is likely that these changes are caused by synaptic mRNAs. Chronic alcohol consumption in mice alters the synaptic expression of mRNA networks (Most, Ferguson et al. 2015) and of microRNAs (Most, Leiter et al. 2016), as we have recently reported. Interestingly, the majority of these changes were isolated to synaptic fractions and were not found in total tissue homogenates. However, the underlying dynamics of this complex synaptic relationship between alcohol, microRNAs and mRNAs remains unclear and the functional importance for alcohol behaviors is yet to be determined.

The primary focus of these experiments was to examine the functional significance of five different synaptic microRNAs – miR-411, miR-203, miR-187, miR-92a and miR-137 – on alcohol consumption in mice. An inhibitor of miR-411 - antagomiR-411, in the prefrontal cortex (PFC), decreased alcohol consumption and preference in alcohol-consuming mice without altering total fluid, saccharin consumption or anxiety related behaviors. Interestingly, antagomiR-411 did not change acquisition of alcohol consumption in mice without a history of alcohol consumption. Furthermore, antagomiR-411 increased the expression of a protein target specifically in neurons, providing a mechanism by which the interaction between synaptic microRNAs and alcohol can affect protein in the synaptic structure, and ultimately, behavior. This study provides a proof of concept that specific synaptic gene networks can predict viable microRNA candidates for in-vivo manipulation of excessive alcohol consumption.

Materials and Methods

Animals

Adult C57BL/6J female mice were obtained from Jackson Laboratory, and maintained at the University of Texas at Austin Animal Resources Center. Female mice were used in these experiments since in this mouse strain they achieve higher levels of alcohol consumption compared to males. Mice were group-housed and given a minimum of two weeks for acclimation in combined housing. Food and water were provided ad libitum and monitored daily, as were the temperature and light/dark cycles. Mice were weighed every four to eight days. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin and adhere to NIH Guidelines for the ethical care and use of animals in research. Mice between the ages of two to six months were used.

Brain Region-Specific microRNA Inhibition

Mice were anesthetized with isoflurane and then placed in a stereotaxic apparatus (model 1900, David Kopf) with a continuous flow of oxygen and isoflurane. The skull was exposed and a burr hole was drilled above the PFC using a drill carbide bit (#73, David Kopf), at the following coordinates relative to bregma: anteroposterior +2.0 mm, mediolateral ±0.5 mm, dorsoventral -2.0 mm. Mice intended for use in molecular studies were at this point injected with treatments, whereas mice intended for alcohol/behavioral experiments were only cannulated but not yet injected.

Injections were performed using a microsyringe (10-µL syringe model #1701, Hamilton) and a 30-gauge needle. Injections constituted either a microRNA LNA inhibitor – AntagomiRs: antagomiR-411, antagomiR-203, antagomiR-187, antagomiR-92a or antagomiR-137 (Exiqon), or a microRNA mimic: mimic-411 or mimic-203 (Dharmacon). The sequences and specific modifications for each treatment are given in Supplementary Table S4.1. The microRNA mimics and antgaomiRs were diluted to 0.5 nmol/µl in artificial cerebrospinal fluid (ACSF; Harvard apparatus). The dose was chosen based

on previous studies using similar methods, such as Jimenez-Mateos et al. 2012 (Jimenez-Mateos, Engel et al. 2012), Mckiernan et al. 2012 (McKiernan, Jimenez-Mateos et al. 2012) and Li et al. 2013 (Li, Li et al. 2013). This dose was 25 times less than that used in Darcq et al. 2014 (Darcq, Warnault et al. 2014), 8 times less than in Teppen et al. 2015 (Teppen, Krishnan et al. 2015), and 2 times less than in Zhu et al. 2007 (Zhu, Liu et al. 2014).

One µl of either a treatment or ACSF (control treatment) was injected over a duration of two minutes. We chose a volume of 1 µl based on the similar volume used by Darcq et al. 2014 (Darcq, Warnault et al. 2014), Li et al. 2013 (Li, Li et al. 2013) and Hollander et al. 2010 (Hollander, Im et al. 2010). After each injection, the injection needle was left in place for one minute before being retracted over a period of two minutes. Incisions were closed with Vetbond, a tissue adhesive (#13479726 Animal Health International).

For cannulation experiments, mice were anesthetized, the skull was exposed and a burr hole was drilled above the PFC as described above. Then 37.5% Phosphoric Acid Gel Etchant (#31297, Kerr) was applied to the skull for 20 seconds and cleaned. Cannulas were glued in place using a cannula holder (#1966) and vetbond. The cannula guide (26 gauge; Plastics One) was inserted 1.5 mm into the prefrontal cortex (see supplementary methods for more detail on the cannulation procedure). OptiBond Solo Plus (#29669, Kerr) was applied and cured using Dental LED curing light (L5-F-ARTB-A1-00-0, Bonart) for one minute. The cannula was then secured using dental cement from the Ortho-Jet Package (#1334PT, Langdental), which was applied according to manufacturer's instructions. The cannula was then capped using a dummy cannula cap, which was 2 mm long (33 gauge; Plastics One).

At the end of either surgery, a preoperative analgesic was administered (5 mg/kg Metacam) (#11247402 Animal Health International) and the mice were placed in a recovery cage until recovery from anesthesia. Cannulated mice were individually housed after surgery and given a one-week recovery period before starting the two bottle choice alcohol paradigm.

Alcohol Consumption

Cannulated mice underwent a two-bottle choice paradigm with a continuous (24-h) access to one bottle of 15% ethanol (referred to as alcohol) and one bottle of water (Lim, Zou et al. 2012), generating levels of consumption similar to previously recorded consumption levels (Osterndorff-Kahanek, Ponomarev et al. 2013, Most, Ferguson et al. 2015). Bottle weights were recorded daily and the amount of alcohol consumed was calculated as g/kg/24hr.

Mice (N=47) were pre-trained to consume 15% alcohol for four weeks, reaching an average alcohol consumption of 12.7 g/kg/day (±2.1) and an average of alcohol preference over water of 0.65 (±0.13). For the first four weeks of consumption, alcohol bottle positions were alternated daily as is the norm in most two-bottle choice paradigms (Blednov, Mayfield et al. 2012). However, in order to measure precise levels of consumption on a daily basis, we determined the preferred position for each mouse and thereafter kept the alcohol bottle in that position for the baseline and treatment periods.

Also, in the experiments where the treatment was given to alcohol-naïve mice, the position switching continued throughout the entire experiment. This is because with no prior alcohol consumption, the alcohol preferred position could not yet be determined.

MicroRNA Treatment Paradigm and Cannula Injections for Alcohol Consuming Mice

After measuring the baseline consumption levels, treatments were infused through the cannulas into the prefrontal cortex of mice. Mice were carefully scruffed and dummy cannulas (cannula cap) were removed. Injectors (2 mm depth) connected to tubing were inserted into the cannulas. The tubing was connected on the other side to a 10 µl syringe and 1 µl of treatment was infused through the cannula for 30-45 seconds. Once finished, the dummy cannula was reinserted and the mice were returned to their home cage. Treatment induced-changes in alcohol consumption, preference and total fluid intake were recorded.

Saccharin Consumption

Mice consumed 0.0165% saccharin in water for four weeks and were then infused with antagomiR-411 or ACSF. The treatment-induced changes in consumption, preference and total fluid intake were recorded for ten days post infusion.

MicroRNA Cannula Infusions for Alcohol Naïve Mice

Mice were cannulated, had a subsequent recovery period of at least one week, and were then infused with antagomiR-411 or ACSF. Three days later, 15% alcohol was introduced in the same two-bottle choice paradigm highlighted above, and the consumption levels, preference and total fluid intake were measured for two weeks.

Open Field Test

Three weeks after antagomiR-411 or ACSF infusions, alcohol-consuming mice were transported to the testing room during the light cycle, one hour prior to testing. Mice were individually placed in the open field and were allowed to freely explore the field while their activity was recorded every 5 minutes for 15 minutes using the Opto-microvarimex animal activity meter (Columbus Instruments) and monitored using 6 light beams placed along the width of the cage at 2.5 cm intervals, 1.5 cm above the floor. The field was covered with a heavy plastic lid with holes for ventilation. After each mouse, the field was cleaned with 10% alcohol, and was left to air out before continuing with the next mouse. Recorded measurements include time, distance and number of entries into the different sections of the field.

Elevated Plus Maze

Three days after the open field test, mice were evaluated for alcohol- and antagomiR-411-induced anxiolysis using the elevated plus maze. Mice were transported to the room one hour prior to testing during the light cycle. Each mouse was individually placed on the central platform of the maze facing an open arm, and allowed to freely explore the maze for 5 minutes during which the

following measurements were recorded: number of open arm entries, number of closed arm entries, total number of entries, time spent in open arms, and time spent in closed arms. A mouse was considered to be on the central platform or any arm when all four paws were within its perimeter. After each mouse, the field was cleaned with 10% alcohol, and was left to air out before continuing with the next mouse.

RNA Isolation

Alcohol-consuming microRNA-treated mice were sacrificed upon completion of behavioral experiments (10 days after cannula infusions of treatment). The alcohol-naïve mice were sacrificed 10 days after injections. All mice were sacrificed by cervical dislocation and decapitated during the light phase of the light/dark cycle, while alcohol was still available to the mice (avoiding any withdrawal effects). Brains were removed, washed with ice-cold saline solution and placed in a coronal Zivic mouse brain slicer with a 0.5 mm resolution (Zivic Instruments). Slices were made at coronal levels Bregma 1-2.5 mm and the PFC was then microdissected, snap frozen in liquid nitrogen and stored in −80°C. Total RNA was extracted using the miRNeasy Mini Kit (#217004, Qiagen) according to the manufacturer's instructions. RNA was then DNAse treated using an RNase-Free DNase Set (#79254, Qiagen). RNA yields and purity were assessed using a NanoDrop 8000 (Thermo Fisher Scientific) and Qubit Fluorometric Quantitation (Thermo Fisher Scientific). RNA quality was determined using the Agilent 2200 TapeStation (ScreenTape #5067-5576; ScreenTape Sample Buffer #5067-5577, Agilent Technologies) with RNA integrity numbers (RIN) above 7.5.

Quantitative Real-Time PCR

To calculate microRNA expression levels, single-stranded cDNA was synthesized from total RNA using reverse transcription PCR (RT-PCR). This was done using the TaqMan® MicroRNA Reverse Transcription Kit (#4366596, Life Technologies) and a primer pool, per manufacturer's instructions, containing miR-411 primer (#001610) and sno-234 primer (#001234). Following the RT-PCR, real-time

quantitative PCR (qPCR) was done in triplicates of 10 µl reactions. Each data point, derived from qPCR assays, represents an average of the replicas. Relative microRNA expression was determined using the ΔΔCT method (Hellemans, Mortier et al. 2007), by calculating the mean difference between the cycle threshold (CT) of the microRNA of interest and the endogenous control (SnoRNA-234) for each sample (ΔCT), and normalizing it to control samples. Reactions were carried out in a CFX384™ Real-Time PCR Detection System (Bio-Rad). For details about the PCR process, see supplementary methods.

Immunohistochemistry and Image Analysis

For immunohistochemistry purposes, tissue fixation was performed. Mice were deeply sedated with isofluorane and then sacrificed with 0.15 ml euthanasia solution (#54925-045, Med Pharmex). Transcardial perfusion was performed using Phosphate Buffer Saline (PBS) and freshly made 4% paraformaldehyde (PFA). Brains were removed and postfixed for 24 hours in 4% PFA at 4°C, and cyroprotected for 24 hours in 20% sucrose in PBS at 4°C. Brains were placed in molds containing OCT compound (VWR) and frozen in isopentane on dry ice. The brains were equilibrated in a -12 to -14°C cryostat (Thermo Fischer Scientific) for at least 1 hour and coronal sections of 30 µm were taken of the PFC and stored in sterile PBS.

See supplementary methods for detailed description of the immunostaining. In short, brain slices were treated with primary antibodies against the proteins of interest, treated with fluorescence-conjugated secondary antibodies, mounted on slides with mounting media and cover slipped. Two sets of control experiments were performed to test specificity: 1) omission of primary antibodies; and 2) omission of secondary antibodies. No immunostaining was detected under either of these conditions. Images were captured (without saturating the signal) using a Zeiss Axiovert 200M Fluorescent Microscope (Zeiss, Oberkocheen) in 20X and 63X magnification, imported into the ImageJ

software package (Schneider, Rasband et al. 2012), and composite images were split into individual channels and overlaid.

To assess the location of the injection/cannula site, the distance of transfection, and the cell type specificity of transfection, fixed brain slices were stained with either a Neun or an Iba antibody or with DAPI. The injection was considered on-target if the needle placement was within 0.5 mm of the desired stereotaxic coordinates. The percent transfection per cell-type was calculated by counting 100 Neun/Iba-positive cells and then looking in the Neun/Iba-antagomiR-411 merged picture to count the number of those cells which were antagomiR-411-positive.

To calculate the effects of treatment on cell-specific GluA2, GABA-B-R1 and GRINA expression, the protein images were first traced using imageJ, then merged with the picture of the cell-type marker (Neun or Iba), and the traces which contained the cell-type of interest were measured for area, integrated density and mean gray value. Approximately 50 cells were counted per brain slice. Background values were collected for each image. The expression was calculated using the equation for corrected total cell fluorescence (CTCF) = Integrated Density – (Area of selected cell X Mean fluorescence of background readings) as done in McCloy et al. 2014 (McCloy, Rogers et al. 2014) and Burgess et al. 2010 (Burgess, Vigneron et al. 2010). This method corrects for background intensity and allows for a normalized comparison between images.

Target Validation

Ten treated brains were flash frozen without dissection in order to confirm the successful targeting of the PFC. These were transferred to a cryostat set at −6° C for 30 minutes before sectioning. Consecutive sections (300 µm) of the brains were sliced, mounted on slides and viewed with a Dual Fluorescent Protein Flashlight (Nightsea). Diffusion of treatment was calculated by the number of slices the treatment was imaged in. On average, four slices per brain contained evidence of treatment, suggesting the diameter of transfection was on average 1.2 mm (data not shown).

Statistical Analysis

QPCR and immunohistochemistry data were analyzed by either a student's t-test or when appropriate, by a two-way analysis of variance (ANOVA). The behavioral data was analyzed using a repeated measures ANOVA. Data from the ANOVA were corrected using Sidak's Post Hoc multiple comparison's correction. Calculated P-values of less than 0.05 were considered statistically significant. Simple calculations were done using Microsoft Office Excel 2013 (Microsoft). Histograms and all statistics and graphics were done using GraphPad Software (Prism).

Results

Development of an Alcohol Consumption Paradigm for Manipulation of microRNAs In-Vivo

We first tested whether the infusion of ACSF itself (our control treatment) can affect the levels of alcohol consumption. Mice consumed alcohol for four weeks, thereafter ACSF was infused into the PFC (Figure 4.1A). Alcohol consumption (Figure 4.1B) and preference (Figure 4.1C) were significantly decreased by 23% and 19% respectively, on the first day post-infusion. Although consumption levels and preference were somewhat reduced for the next four days, they were not significantly different from baseline levels, and returned to baseline within that four day period. Total fluid consumption did not change (Figure 4.1D). These results suggest that the experimental model is suitable for testing microRNA treatment effects on alcohol consumption and preference, but that the effects of the injection itself may confound any treatment effects during the first day post infusion.

We asked whether a second injection in the same mice is possible, and whether the alcohol consumption and preference response is similar to the first injection. Therefore, ACSF was infused a second time in the same mice. There were no significant differences in consumption levels or preference between the first and second infusions (see Figure 4.1). These results suggest that injecting the same mice with ACSF a second time is possible and will not alter the consumption levels or preference between injections.

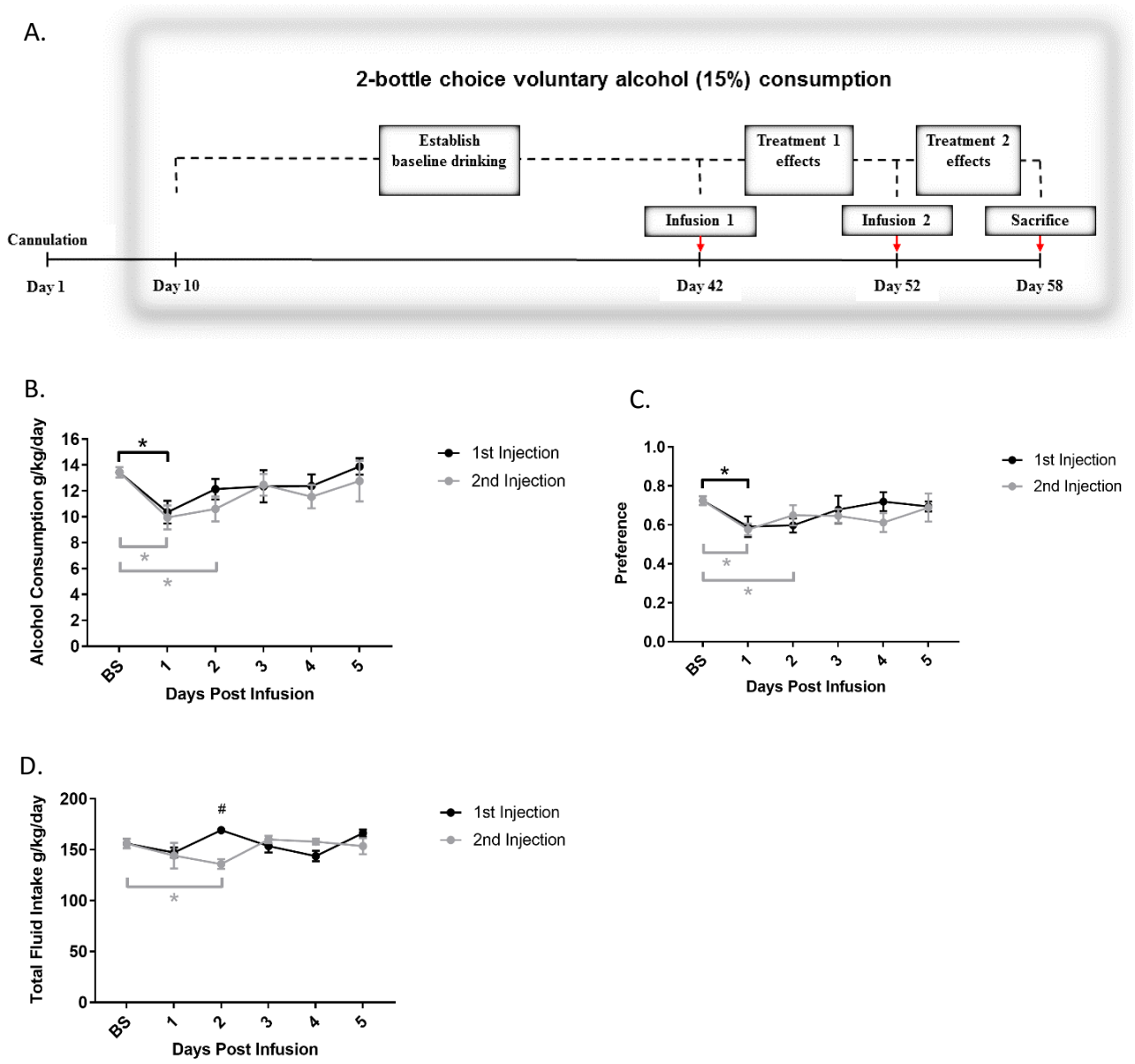


FIGURE 4.1: INFUSION OF ACSF DOES NOT CHANGE ALCOHOL CONSUMPTION

A. Timeline of the two-bottle choice alcohol consumption paradigm relative to treatment cannula infusion – a within subject analysis. Mice were cannulated and had a ten-day recovery period. Alcohol was then introduced in a two-bottle choice paradigm for four weeks. Side preference for the alcohol bottle was determined for ten days. Baseline (BS) levels of consumption, preference of alcohol over water and total fluid intake were measured for three to four days. We then infused treatments/ACSF into the PFC cannulas of the mice, and treatment effects were measured for five to ten days. For the reinfusion experiments, mice were re-infused with treatment/ACSF and the alcohol consumption was measured for another six to eight days. B. Alcohol consumption (g/kg/day) for the days before and after ACSF infusion for the first and second injections. BS is calculated by averaging the consumption levels from the

three days prior to injection. A repeated measures Analysis of Variance (ANOVA) by both factors revealed a significant treatment day effect (P=0.0002), stemming from differences in consumption levels between BS and day 1 for the first injection (P=0.0149) and between BS and day 1 and day 2 for the second injection (P=0.0047, P=0.0288, respectively). Although lower consumption levels were seen during the next four days, none of these days were significantly different from BS. No differences were seen between first and second injections for any of the days. For all panels, N=8, * denotes significance (P<0.05, determined by Sidak's post hoc analysis) for the treatment day effects, in black for first injection, in gray for second injection and # denotes significance between treatments. Error bars are given as standard error of the means. C. Preference for alcohol over water (shown as a ratio) for the days before and after ACSF infusion. Repeated measures ANOVA by both factors revealed a significant treatment day effect (P=0.0044), stemming from differences in preference levels between BS and day 1 and day 2 for the first injection (P=0.0259, P=0.0377, respectively), and between BS and day 1 for the second injection (P=0.0111). D. Total fluid intake for the days before and after ACSF infusion. Repeated measures ANOVA by both factors revealed a significant day by treatment interaction (P=0.0014), stemming from differences between first and second injections on day 2 (P=0.0005) and a difference between BS and day 2 for the second injection (P=0.0493).

Knockdown of miR-411 Decreases Alcohol Consumption in Alcohol Consuming Mice

We recently showed that alcohol consumption changes the levels of miR-411, miR-203, miR-92a, miR-187 and miR-137 in the synapse (Most, Leiter et al. 2016). We therefore selected these five microRNAs for manipulation. We hypothesized that manipulations of microRNA levels in the opposite direction produced by chronic alcohol consumption should oppose the neuroadaptations caused by alcohol and reduce alcohol consumption. We began by studying the effects of miR-411 manipulation in alcohol-consuming mice with either antagomiR-411 (N=13), mimic-411 (N=15) or ACSF (N=14). Alcohol consumption was markedly decreased following miR-411 knockdown (by antagomiR-411) compared to ACSF (Figure 4.2A; Supplementary Figure S4.1A), as well as preference for alcohol over water (Figure 4.2B). However, there was no change in consumption or preference with mimic-411 compared with ACSF. There were no differences between the three treatments in total fluid consumption (Figure 4.2C, Supplementary Figure S1b). Observing the distribution of the changes in alcohol consumption on post infusion days, we determined that the population response to antagomiR-411 was significantly different from that of ACSF (Figure 4.2D).

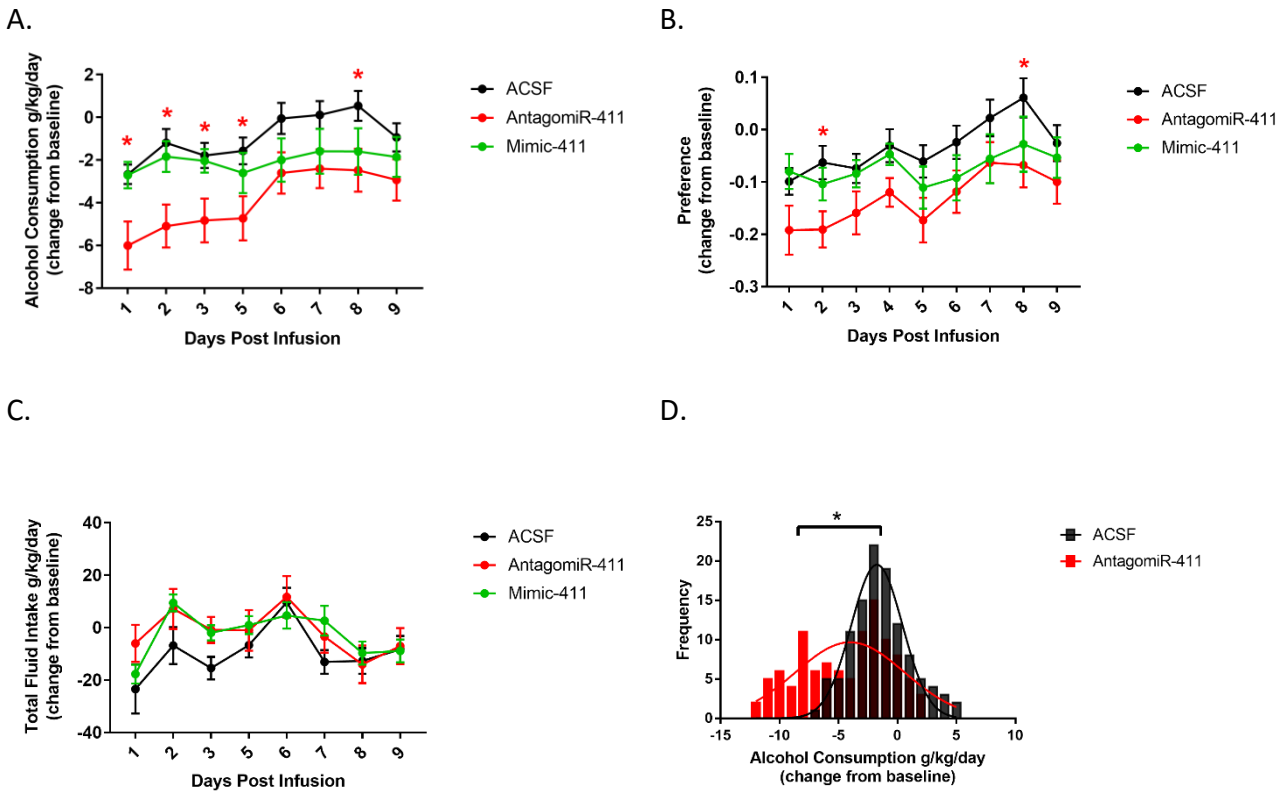


FIGURE 4.2: ANTAGOMIR-411 DECREASES ALCOHOL CONSUMPTION IN ALCOHOL CONSUMING MICE

A. The effects of antagomiR-411 or mimic-411 on alcohol consumption. Data is presented as change in consumption levels from baseline (BS) per each day (BS - day X), in response to treatment with antagomiR-411 (N=13), mimic-411 (N=15) or ACSF (N=14). A repeated measures analysis of variance (ANOVA) showed a main effect of treatment (P=0.0132) and a main effect of time (P<0.0001). AntagomiR-411 reduces alcohol consumption compared to ACSF (day 1 P=0.0137; day 2 P=0.0033; day 3 P=0.0269; day 5 P=0.0208; day 8 P=0.0289). Mimic-411 does not change alcohol consumption compared to ACSF. For all panels, * denotes significance (P<0.05, determined by Sidak's post hoc analysis) and error bars are given as standard error of the means. In day 4, mouse weights were measured, thus the data from that day was not included in the analysis or graphs. B. Change in preference from BS in response to treatment. A repeated measures ANOVA showed a main effect of treatment (P=0.0477) and a main effect of time (P<0.0001). AntagomiR-411 significantly reduced the preference for alcohol compared to ACSF (day 2 P=0.0299; day 8 P=0.0291). C. Change in total fluid consumption from BS in response to treatment. None of the treatments changed total fluid consumption compared to ACSF, though there was a main effect of time (P<0.0003). D. Histogram of the change in consumption levels in response to treatment with antagomiR-411 and ACSF, collapsed throughout the days post infusion. AnatgomiR-411 reduces alcohol consumption (mean = -3.88 ± 1.02) and a t-test (unequal variance) shows this is significantly different (P=2.42E-10) from ACSF (mean =3.75 ± 2.57).

We next tested whether the change in consumption levels was specific to manipulation of miR-411 or if it generalizes to manipulation with other alcohol-responsive microRNAs. In a different group of mice, we infused antagomiR-203 (N=5), mimic-203 (N=5) or ACSF (N=5) into the PFC of

chronically consuming mice and measured the changes in consumption levels, preference and total fluid intake. Neither treatment with antagomiR-203 nor treatment with mimic-203 changed alcohol consumption (Figure 4.3A), preference (Figure 4.3B) or total fluid intake (Figure 4.3C) compared to baseline or ACSF treatment.

In a different group of mice, we tested the effects of knockdown of miR-92a, miR-187 or miR-137 on alcohol consumption, by infusing either antagomiR-92a (N=13), antagomiR-187 (N=8), antagomiR-137 (N=18) or ACSF (N=31), into the PFC of alcohol-consuming mice. However, neither of these treatments were effective in changing the consumption levels (Figure 4.3D), preference (Figure 4.3E), or total fluid intake (Figure 4.3F). We then studied the effects of a second treatment infusion in these mice. A cocktail of all three microRNAs (miR-92a, miR-187 and miR-137) (N=9), or antagomiR-411 was infused into the PFC (N=12) and compared to ACSF infusion (N=8), to test the hypothesis that manipulation of a combination of microRNAs may be more effective than single microRNA manipulation. However, the microRNA cocktail was not effective in changing alcohol consumption or preference (data not shown) regardless of which treatment was given previously. In contrast, infusion of antagomiR-411 after treatment with ASCF or antagomiR-92a produced a reduction in alcohol consumption and preference, similar to the previous results with antagomiR-411 (data not shown). There were no significant differences between ACSF+antagomiR-411 infusion and antagomiR-92a+antagomiR-411 infusion. Overall, this data suggests that the effects on alcohol consumption seen with antagomiR-411 are specific to miR-411, and do not generalize to miR-203, miR-92a, miR-187 and miR-137.

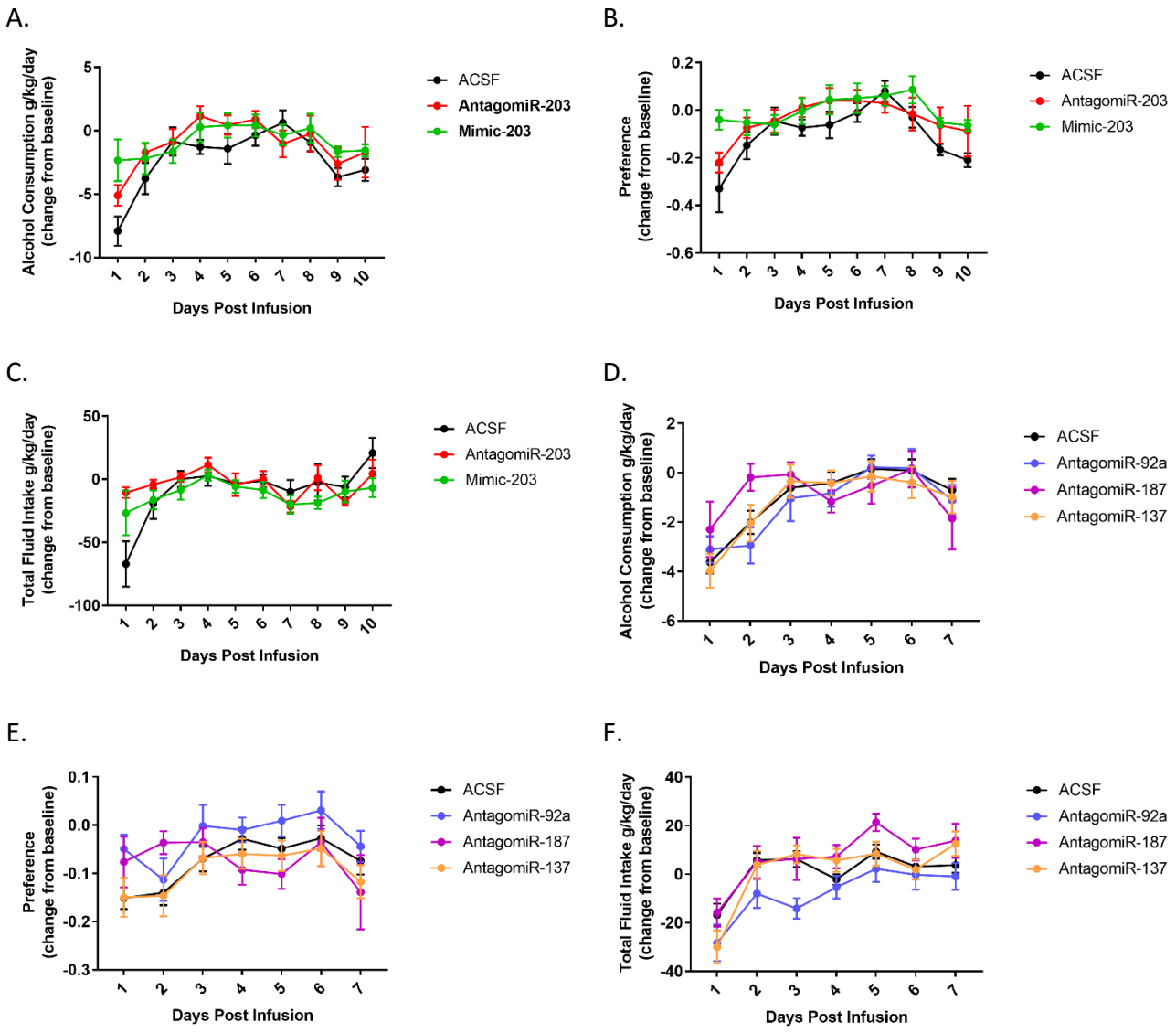


FIGURE 4.3: MANIPULATION OF MIR-203, MIR-92A, MIR-187 OR MIR-137 DOES NOT CHANGE ALCOHOL CONSUMPTION

A. The effects of miR-203, miR-92a, miR-187 or miR-137 on alcohol consumption. Data is presented as change in consumption levels from baseline (BS) per each day (BS - day X), in response to treatment with antagomiR-203, mimic-203 or ACSF (N=5 for all three groups). A two-way repeated measures Analysis of Variance (ANOVA) revealed that neither treatment changed alcohol consumption, preference or total fluid intake. There was a main effect of time (day post infusion, $P<0.0001$) for all three measures. For all panels, significance is denoted by $P<0.05$ and is determined by Sidak's post hoc analysis, and error bars are given as standard error of the means. B. Preference for alcohol over water. C. Total fluid intake. D. Change in consumption levels from BS in response to treatment with antagomiR-92a (N=13), antagomiR-187 (N=8), antagomiR-137 (N=18) or ACSF (N=31). A two-way repeated measures ANOVA revealed that neither antagomiR-92a, antagomiR-187 nor antagomiR-137 changed alcohol consumption, E. preference or F. total fluid intake compared to control. There was a main effect of time ($P<0.0001$) for all three measures.

Knockdown of miR-411 Does Not Change Saccharin Consumption

Because manipulation of miR-411 was successful in decreasing alcohol consumption, we determined whether miR-411 knockdown effects are specific to alcohol or generalize to another reward, by examining the effects of antagomiR-411 (N=12) on saccharin consumption and comparing them to ACSF (N=10). AntagomiR-411 did not change saccharin consumption (Figure 4.4A) nor preference (Figure 4.4B). There were no differences in total fluid consumption among the three groups (Figure 4.4C). Thus, the effects of miR-411 knockdown on alcohol consumption and preference do not alter the propensity to consume another rewarding solution.

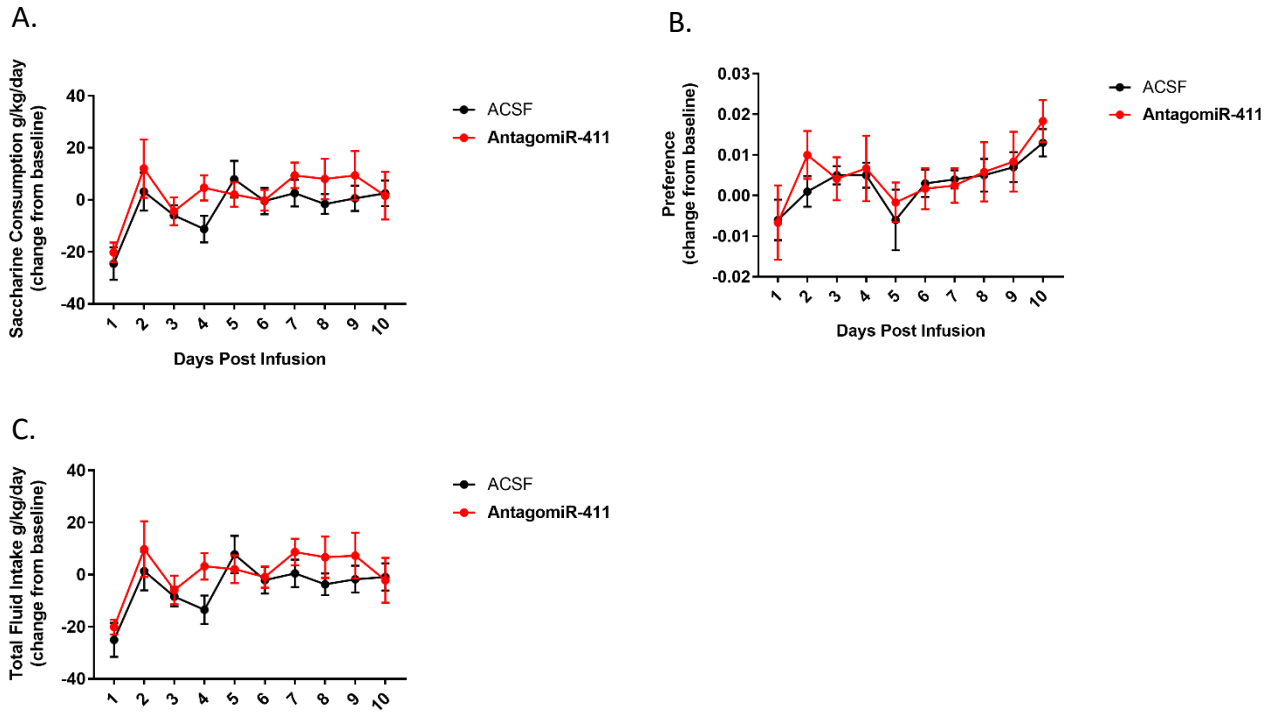


FIGURE 4.4: ANTAGOMIR-411 DOES NOT CHANGE SACCHARIN CONSUMPTION

A. The effects of antagomiR-411 on saccharin consumption levels, B. preference for saccharin over water and C. total fluid intake. Data is calculated as change from baseline (BS) per each day (BS - day X) in response to treatment with antagomiR-411 or ACSF infusion (N=10-12). A two-way repeated measures Analysis of Variance (ANOVA) revealed that neither treatment changed saccharin consumption, preference or total fluid intake. There was a main effect of time (day post infusion, $P<0.0001$) for all three measures.

For all panels, significance is denoted by $P<0.05$ and is determined by Sidak's post hoc analysis and error bars are given as standard error of the means.

Knockdown of miR-411 Does Not Change Alcohol Consumption in Mice Without a History of Alcohol Consumption

Because antagomiR-411 caused a decrease in alcohol consumption and preference when given after chronic alcohol consumption, we asked whether antagomiR-411 can affect the acquisition of alcohol consumption in alcohol-naïve mice. We infused antagomiR-411 (N=14) or ACSF (N=10) into the PFC of alcohol-naïve mice and then introduced 15% alcohol in a two-bottle choice paradigm. AntagomiR-411 did not change levels of alcohol consumption (Figure 4.5A) and preference (Figure 4.5B). Total fluid intake also remained constant (Figure 4.5C).

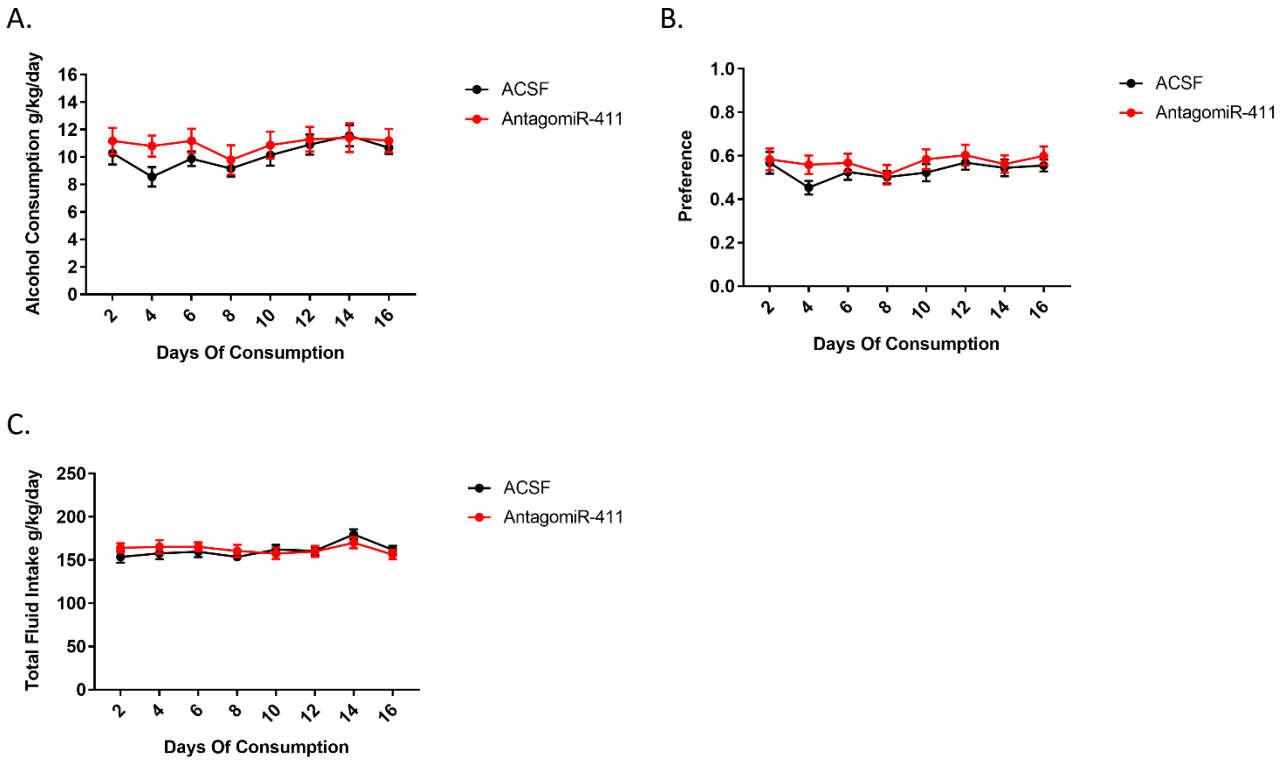


FIGURE 4.5: PRETREATMENT WITH ANTAGOMIR-411 DOES NOT CHANGE ALCOHOL CONSUMPTION IN ALCOHOL-NAÏVE MICE

A. The effects of antagomiR-411 on acquisition of alcohol consumption. Alcohol consumption levels (g/kg/day) for antagomiR-411 treated mice (N=14) and ACSF treated mice (N=10). A repeated measures analysis of variance (ANOVA) revealed a main effect of time ($P=0.0005$), but no significant effect of treatment nor an interaction between the two. For all panels, significance is denoted by $P<0.05$ and is determined by Sidak's post hoc analysis, and error bars are given as standard error of the means. B. Preference for alcohol over water. A repeated measures ANOVA revealed a main effect of time ($P=0.0108$), but no significant effect of treatment nor an interaction between the two. C. Total fluid intake. A repeated measures ANOVA revealed a main effect of time ($P<0.0001$), and a significant interaction effect (time X treatment, $P=0.0032$), but there was no significant effect of treatment.

Knockdown of miR-411 Does Not Change Stress-Related Behaviors

Stress is known to alter alcohol consumption (Lopez, Anderson et al. 2016). We therefore investigated whether miR-411 manipulation altered two different behaviors sensitive to stress, as a control for treatment's effects on alcohol consumption. Elevated plus maze and open field test were performed on alcohol-consuming mice after infusion of antagomiR-411 (N=10) or ACSF (N=8). In the

open field test, distance traveled, resting time, horizontal and vertical counts, center and total events, and time in center were not different between antagomiR-411 and ACSF (Figure. 4.6A). The same was true in the elevated plus maze, where number, distance and duration of entries into each arm were recorded, showing no differences between antagomiR-411 and ACSF in any of the measures (Figure 4.6B).

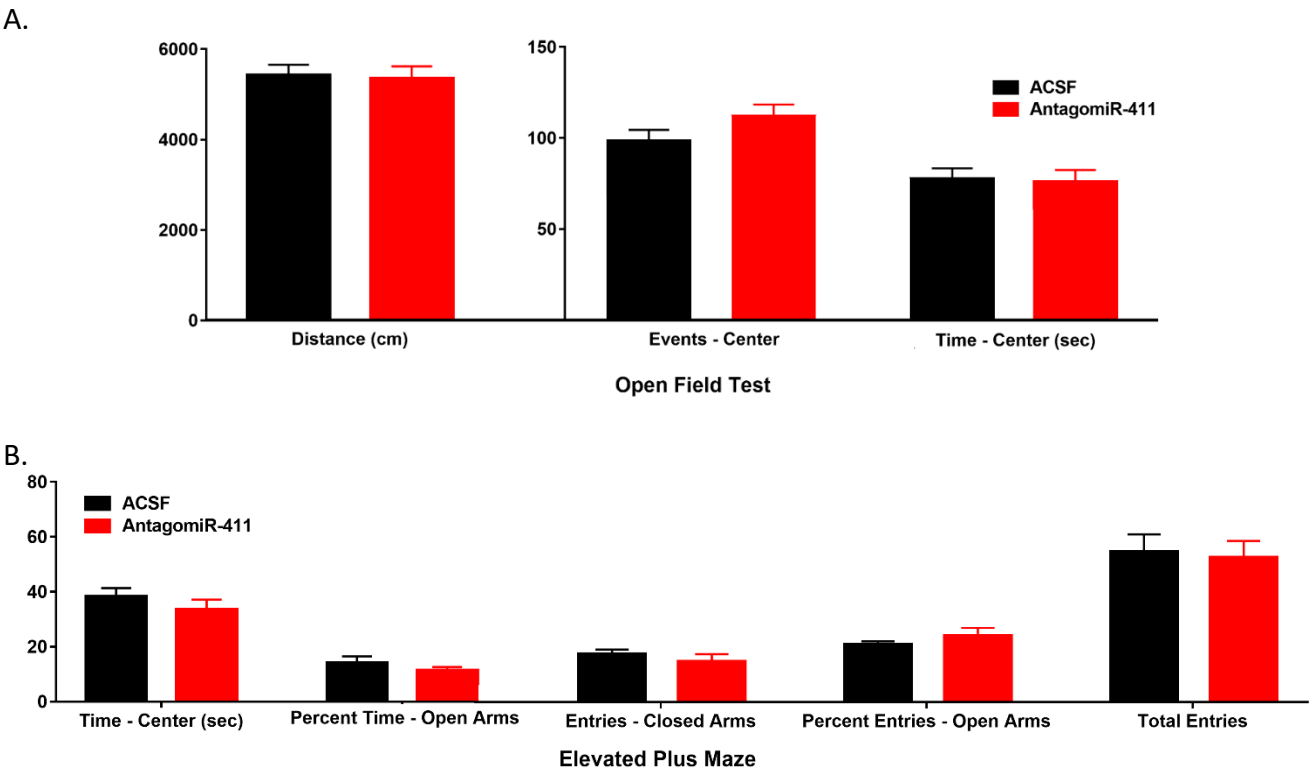


FIGURE 4.6: ANTAGOMIR-411 DOES NOT CHANGE BEHAVIORAL RESPONSES IN THE ELEVATED PLUS MAZE OR OPEN FIELD TESTS

A. Effects of antagomiR-411 on anxiety-related behaviors. Response to open field test after treatment with antagomiR-411 (N=10) or ACSF (N=8). A t-test revealed there were no differences between the groups. For all panels, significance is determined by $P<0.05$ and error bars are given as standard error of the means. B. Response to elevated plus maze after treatment with antagomiR-411 or ACSF. A t-test revealed there were no differences between the groups.

AntagomiR-411 is Functionally Active in the Cell, as seen by the Increase in a Target Protein After Treatment.

We first confirmed that the cannulations were in the correct location (Figure 4.7A, B and C). AntagomiR-411 was found in neurons and microglia - 98% of Neun-positive (neurons, Figure 4.7D), 86% of Iba-positive (microglia, Figure 4.7E) were transfected with antagomiR-411.

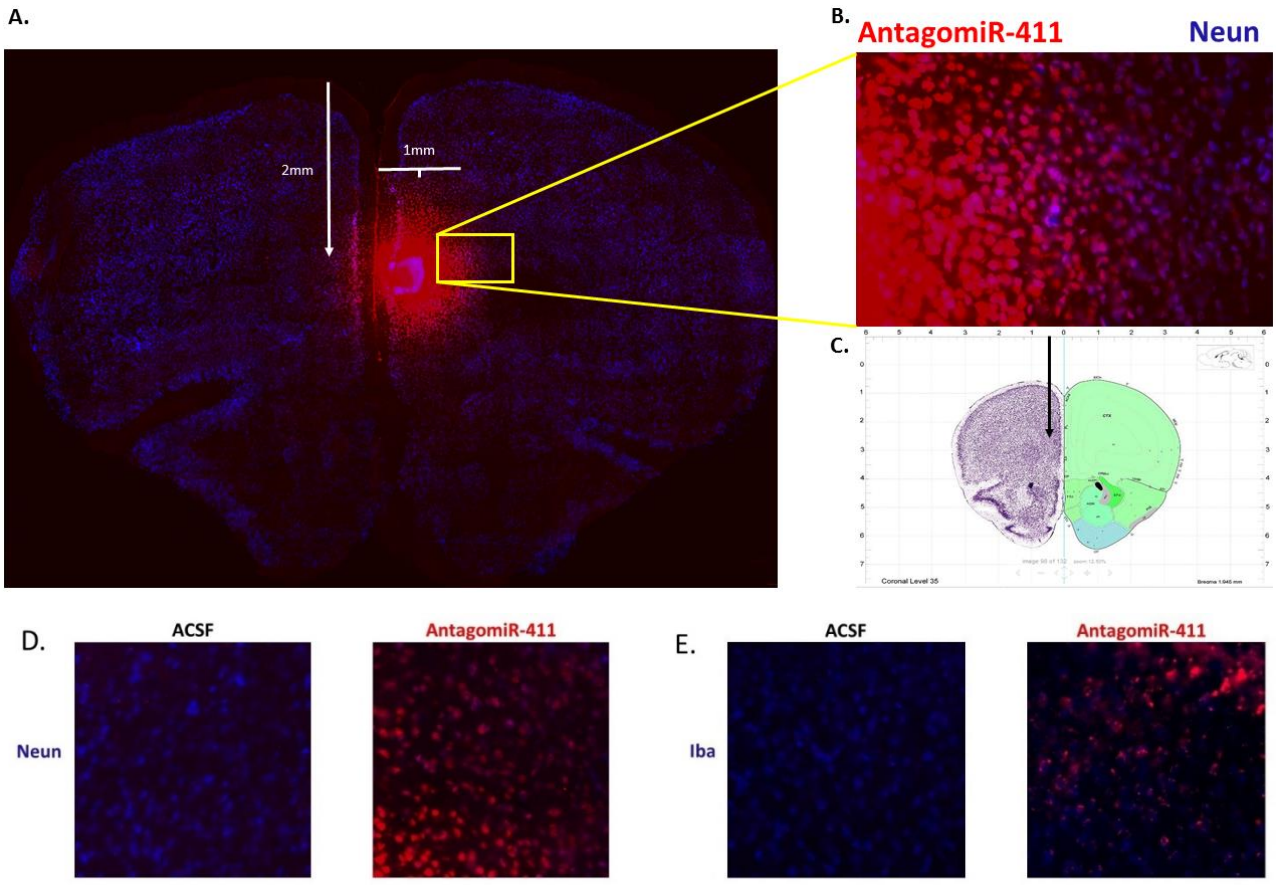


FIGURE 4.7: ANTAGOMIR-411 TRANSFECTS BOTH NEURONS AND MICROGLIA

A. Transfection of antagomiR-411 in the PFC. Representative image of a PFC slice from an antagomiR-411 treated alcohol-consuming mouse. AntagomiR-411 is shown in red and neurons (Neun-labeled cells) are shown in blue. The injection site was at the following coordinates relative to bregma: anteroposterior +2 mm, mediolateral ±0.5 mm, dorsoventral -2.0 mm. B. Magnified image of an area proximal to the injection site. C. Representative atlas mapping of the antagomiR-411 injection site within the PFC (denoted by an arrow), (image taken from Allen's brain mouse reference atlas). D. Cell type-specific transfection with antagomiR-411 (red), in neurons (Neun-labeled cells, blue) and E. Microglia (Iba-labeled cells, blue), from an alcohol-consuming antagomiR-411-treated mouse (right) compared to an ACSF treated mouse (left).

We then measured the levels of miR-411 in response to antagomiR-411 or mimic-411, in chronically consuming mice compared to alcohol-naïve mice. Levels of miR-411 were reduced in response to treatment with antagomiR-411 (Figure 4.8A) and were increased after treatment with mimic-411 (Figure 4.8B), compared to the ACSF group, and this was true for both the alcohol-exposed and control mice.

We measured the change in expression of a predicted target of miR-411 (Supplementary Figure S4.2.), the GluA2 subunit of the AMPA receptor, after treatment with antagomiR-411, and found that levels of GluA2 protein were increased in response to treatment with antagomiR-411, specifically in Neun-labeled cells (Figure 4.8C). This was not the case for GRINA (Figure 4.8D) and GABA-B-R1 (Figure 4.8E), two proteins which were not predicted to be targeted by miR-411 (Figure 4.8F). These results suggest that a miR-411, a microRNA that decreases GluA2 levels in the PFC, plays a role in regulating both preference and consumption of alcohol in alcohol consuming mice.

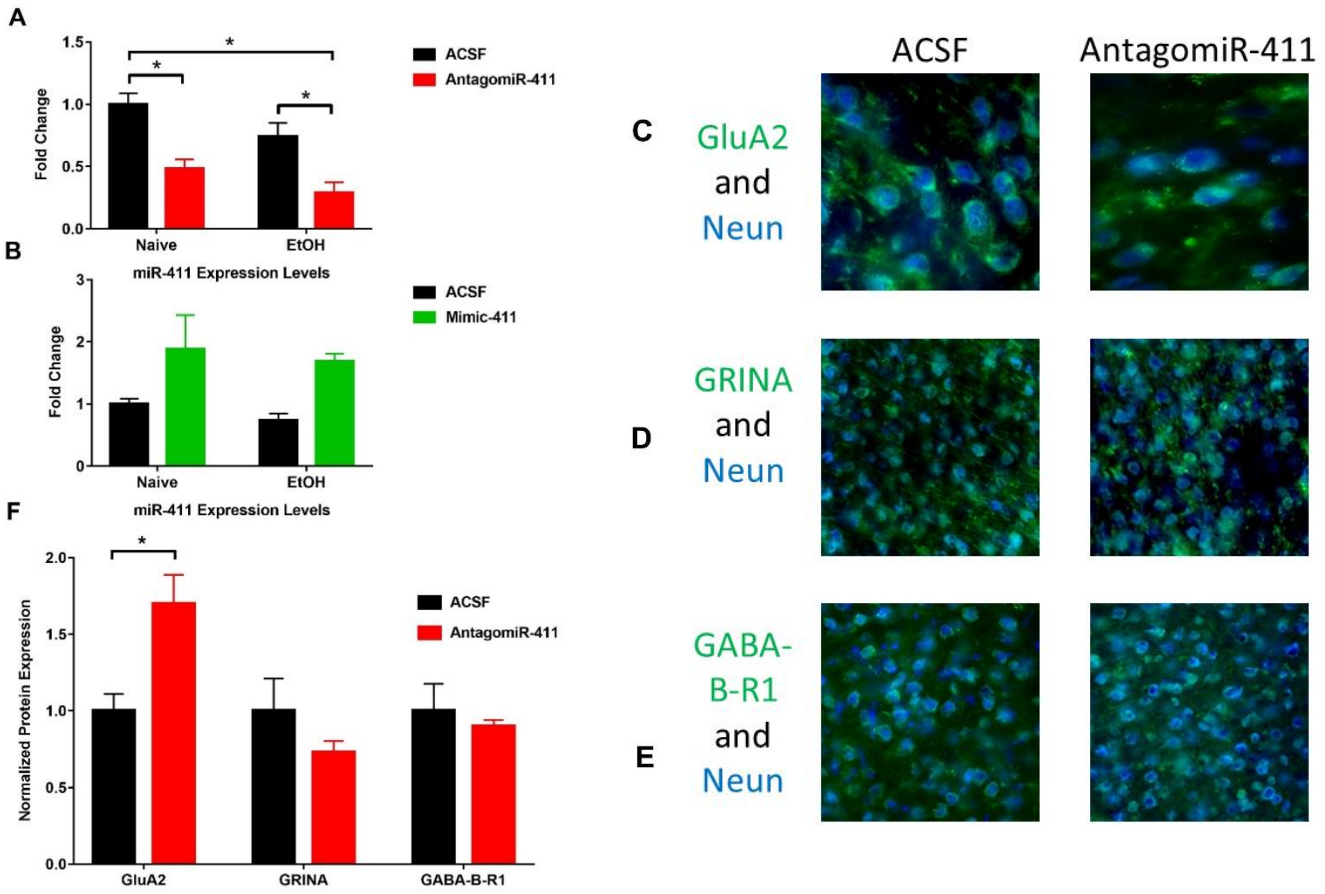


FIGURE 4.8: ANTAGOMIR-411 DECREASES MIR-411 EXPRESSION AND INCREASES GLUA2 PROTEIN LEVELS

A. MiR-411 expression levels in response to treatment with antagomiR-411. Levels of miR-411 as measured with qPCR of samples: ACSF (N=10), antagomiR-411 (N=8), alcohol-ACSF (ACSF-EtOH, N=8) and alcohol-antagomiR-411 (antagomiR-411-EtOH, N=6). QPCR was quantified using the ΔΔCt method (relative to ACSF naïve levels). Sno-RNA-234 was used as an endogenous control. A two-way Analysis of Variance (ANOVA) revealed antagomiR-411 significantly reduced miR-411 levels (P=<0.0001) and alcohol significantly reduced miR-411 levels (P=0.0196). This stemmed from differences between ACSF and antagomiR-411 (P=0.0015), ACSF-EtOH and antagomiR-411-EtOH (P=0.0147), and ACSF and antagomiR-411-EtOH (<0.0001). For all panels, significance (P<0.05) is denoted by *, and is determined by Sidak's post hoc analysis. Error bars are given as standard error of the means. B. Levels of miR-411 as measured with qPCR of samples: ACSF (N=9), mimic-411 (N=6), alcohol-ACSF (ACSF-EtOH, N=8) and alcohol-mimic-411 (mimic-411-EtOH, N=6). A two-way ANOVA revealed mimic-411 significantly increased miR-411 levels (P=0.0011). None of the specific comparisons were significant, though there was a trend towards a significant difference between ACSF and mimic was (P=0.0504). C. Expression levels of GluA2 in Neun-labeled cells in the PFC. Representative X63 merged images of a GluA2 labeled slice (green) merged with an image of a Neun labeled slice (blue) from an alcohol-antagomiR-411 slice (right), compared to an alcohol-ACSF slice (left). D. Representative X20 merged images of a GABA-B-R1 labeled slice (green) and E. GRINA labeled slice (green) merged with an image of a Neun labeled slice (blue). There were no differences in levels of GABA-B-R1 or GRINA, between alcohol-antagomiR-411 and alcohol-ACSF. F. Average cell fluorescence for GluA2, GABA-B-R1 and GRINA, as calculated with the Corrected Total Cell fluorescence (CTCF) method. A t-test revealed the levels of GluA2 were higher after treatment with antagomiR-411 compared to ACSF (P=0.0142).

Discussion

Chronic alcohol consumption alters the expression of many microRNAs and mRNAs in the brain, providing a basis for long-lasting neuroadaptations. However, the specific microRNAs involved in the transition from chronic consumption to alcoholism, and the processes by which these microRNAs target mRNAs to affect cellular function and behavior are not well understood. Here we show that chronic alcohol consumption alters the expression of miR-411 and GluA2 protein in the PFC of chronically consuming mice. Silencing miR-411 activity decreases alcohol consumption and preference in chronically consuming mice, while simultaneously increasing miR-411 target-protein levels. This is in contrast to the effects of silencing of miR-203, miR-92a, miR-187 and miR-137, which did not have any apparent effects on alcohol consumption or preference. We also show that miR-411 is selective for alcohol consumption and does not change saccharin consumption, locomotion or anxiety related behaviors. Importantly, we show that silencing miR-411 caused a reduction in alcohol consumption in already consuming mice, but did not affect the acquisition of consumption in mice without a history of alcohol consumption. These results suggest that miR-411 is specifically involved in the neuroadaptations after long-term alcohol use (see Table 4.1 for a summary of the molecular and behavioral effects of miR-411 manipulation).

Behavioral paradigm	Molecular effects	Behavioral effects
30 days of alcohol consumption compared to water consumption.	Reduces miR-411 in the PFC.	Alcohol consumption and preference are maintained.
AntagomiR-411 infusion after 30 days of alcohol consumption.	Reduces miR-411 in the PFC. Increases expression of miR-411 target-protein GluA2.	Decreases alcohol consumption and preference. Does not change total fluid intake.
Mimic-411 infusion after 30 days of alcohol consumption.	Increases miR-411 in the PFC.	Alcohol consumption and preference are not changed.
AntagomiR-411 infusion after 30 days of saccharin consumption.	NA	Saccharin consumption and preference are not changed.
AntagomiR-411 infusion before acquisition of alcohol consumption.	Reduces miR-411 in the PFC.	Alcohol consumption and preference are not different between treatment group and control.

TABLE 4.1: SUMMARY OF THE MOLECULAR AND BEHAVIORAL EFFECTS OF MIR-411 MANIPULATION

MiR-411 Knockdown by Alcohol may be a Homeostatic Adaptation to Oppose Alcohol Consumption

The traditional view of the link between microRNA levels and behavior claims that if alcohol consumption (the behavior) decreases levels of a microRNA, then in order to reduce alcohol consumption (reversing the behavior), one needs to increase the microRNA (i.e. restore the microRNA to its original levels). However, our results show the opposite: Alcohol consumption decreased the levels of miR-411, but then our further reduction of levels of miR-411 led to a reduction in alcohol consumption. These results suggest that changes in microRNA levels produced by alcohol consumption may not promote consumption, but are rather a homeostatic adaptation, and actually oppose consumption. This has shown to be the case for miR-30a (Darcq, Warnault et al. 2014), miR-206 (Tapocik, Barbier et al. 2014) and miR-124 (Bahi and Dreyer 2013). However, there are reports of the opposite, in which neuroadaptations resulting from drug use subsequently encourage further

consumption, and restoring the microRNA levels back to normal reduces the behavior. This relationship between effects of drugs on microRNA levels and the effects of microRNA manipulations on drug consumption has been seen with miR-382 (Li, Li et al. 2013), miR-212 (Hollander, Im et al. 2010, Im, Hollander et al. 2010), miR-124, let-7d and miR-181 (Chandrasekar and Dreyer 2011). Drug-induced neuroadaptations and transcriptional changes influence a complex regulatory network. One possibility is that alcohol only directly decreases mRNA expression (specifically mRNAs targeted by miR-411), and that the cellular response is to decrease the levels of miR-411 in order to cause an increase in the levels of its targets and bring the cell back to homeostasis (Nunez, Truitt et al. 2013). This 'counter-adaptivity' explanation makes sense in light of seeing no changes in consumption levels when using antagomiR-411 in alcohol-naïve mice. Future studies are warranted in order to reveal the exact order of the molecular cascade in response to alcohol, and how this is changed when manipulating a microRNA.

AntagomiR-411 Decreases Alcohol Consumption in Chronically Consuming Mice

We saw that treatment with antagomiR-411 in chronically consuming mice reduces subsequent alcohol consumption and that this effect was not seen in the saccharin consumption experiment. One potential explanation is that reduction of miR-411 expression levels is rewarding and that this reward can replace the alcohol-consumption induced-reward, thus reducing the need to consume alcohol. However, this reward is specific to alcohol and does not involve saccharin. Thus, it may be necessary to first induce dependence on a specific rewarding substance such as alcohol, before this reward can be replaced with a different type of reward such as antagomiR-411. Further experiments are necessary in order to determine if miR-411 expression levels directly control reward. For example: the use of conditioned place preference with antagomiR-411 in order to see if it is sufficiently rewarding to condition a mouse to a certain place in the cage; using an intracranial self-

stimulation test (ICSS) to observe if antagomiR-411 can reduce the reward threshold of the stimulation.

Knockdown of miR-411 Reduces Alcohol Consumption whereas Increased miR-411 Does Not Accomplish the Opposite

Although antagomiR-411 decreased alcohol consumption and preference, administering mimic-411 did not change alcohol consumption or preference. There are a few possible explanations why this may have occurred. One reason may be that because female C57BL/6J mice display already high levels of drinking, a 'ceiling effect' may make it difficult to further increase the consumption levels with mimic-411. A second reason could be that the key mRNA targets of miR-411 are at low cellular levels (i.e. downregulated by alcohol or bound by other microRNAs/binding proteins), such that increasing the expression of miR-411 has no consequences on the molecular functional output of the RNA silencing machinery. This theory goes hand in hand with the 'counter-adaptivity' hypothesis, where alcohol directly reduces the expression of miR-411 targets, and in turn reduces the expression of miR-411 to prevent it from further decreasing the expression levels of its targets.

Manipulation of Four Other microRNAs Did Not Change Consumption

In order to determine if other microRNAs affect alcohol consumption, we manipulated five key alcohol-responsive microRNAs in the PFC of mice: miR-411, miR-203, miR-92a, miR-187 and miR-137. These microRNAs were selected based on changes produced by alcohol treatments in other studies: in humans - miR-203 and miR-92a (Lewohl, Nunez et al. 2011); in rats - miR-187 and miR-137 (Tapocik, Solomon et al. 2013); and in mice - miR-187 (unpublished data). These microRNAs were also altered in the amygdala of mice – miR-411 in the total homogenates (Most, Leiter et al. 2016); miR-203, miR-187 and miR-137 in the synaptoenurosomes (Most, Leiter et al. 2016), and in non-PFC cell cultures - miR-203 (Van Steenwyk, Janeczek et al. 2013). Here we show that, the manipulation of these other four microRNAs did not change alcohol consumption levels or preference, whereas

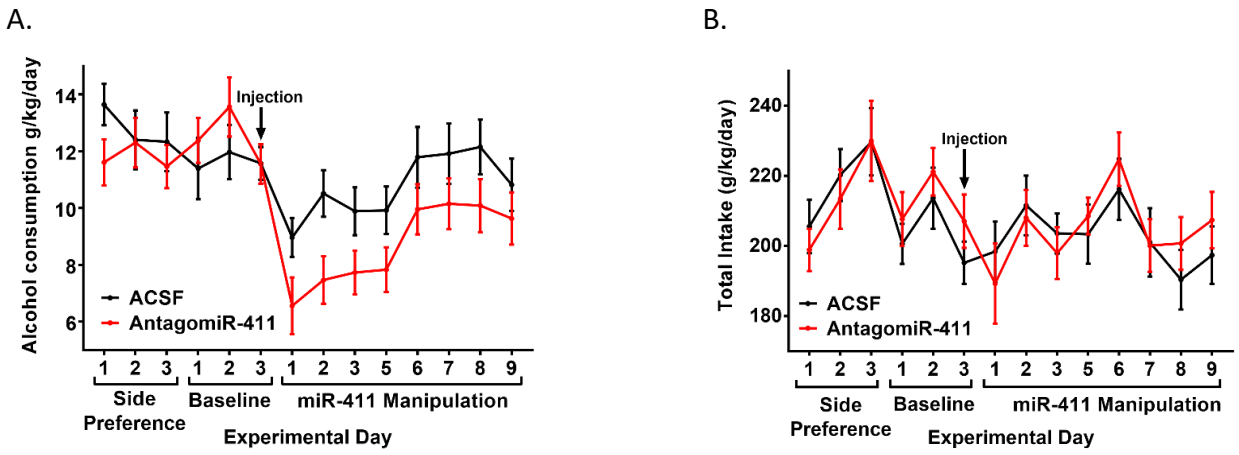
manipulation of miR-411 did manage to change consumption levels and preference significantly. One possibility is that different microRNAs are involved in different periods of alcohol dependence, such that different time points are necessary for the manipulation of these microRNAs to take an effect. One example is from Im et al. who increased the levels of miR-212 and showed that cocaine intake is markedly lower in miR-212 rats with extended access to cocaine, but not in rats with restricted access to cocaine (Im, Hollander et al. 2010).

Summary

MicroRNAs are clearly involved in the neuroadaptive responses induced by exposure to substances of abuse, and their large number of targets encompass a dynamic regulatory network. Because a single microRNA targets many mRNAs, drugs of abuse can effectively hijack a complex network. If microRNAs live up to their role as master regulators suggested here and elsewhere, then their impact on drug-mediated responses and therapeutic strategies will be of clinical importance.

Supplementary Material

Supplementary Figures



SUPPLEMENTARY FIGURE S4.1: ANTAGOMIR-411 REDUCES ALCOHOL CONSUMPTION AND PREFERENCE WITHOUT CHANGING TOTAL FLUID INTAKE

A. Effects of miR-411 manipulation on alcohol consumption levels and on B. total fluid intake, throughout the experimental days, including bottle position preference days (side preference) and baseline measurements.

```
mmu-miR-411   3' gcaugcgauaugccAGAUGAu 5'
                                |||||
      Gria2   5' acagagcauccuuuUCUACUu 3'
```

SUPPLEMENTARY FIGURE S4.2: LINING OF MIR-411 ON THE 3'UTR OF TARGET GENE GLUA2 (GRIA2)

Target prediction data and image have been taken from www.miRNA.org (Enright, John et al. 2003, John, Enright et al. 2004, Betel, Wilson et al. 2008, Betel, Koppal et al. 2010).

Supplementary Tables

AntagomiR/Mimic	Product number	Vendor	Sequence
AntagomiR-411-5p	1999998	Exiqon	GCTATACGGTCTACT
AntagomiR-203-3p	1999998	Exiqon	GGTCCTAAACATTTCA
AntagomiR-92a-3p	1999998	Exiqon	CGGGACAAGTGCAAT
AntagomiR-187-3p	1999998	Exiqon	TGCAACACAAGACACG
AntagomiR-137	1999998	Exiqon	GCGTATTCTTAAGCAA
Mimic-411-5p	C-121503-00-60	Dharmacon	UAGUAGACCGUAUAGCGUACG
Mimic-203-3p	C-121502-00-60	Dharmacon	GUGAAAUGUUUAGGACCACUAG

SUPPLEMENTARY TABLE S4.1: SEQUENCES AND MODIFICATIONS FOR THE ANTAGOMIRS AND MIMICS USED IN THE STUDY

All antagomiRs were HPLC purified and 5' TYE-563 labeled. All mimics were HPLC purified and 5'DY547-psngr labeled.

Supplementary Methods

Cannulas properties: Guide cannulas (Plastics One) were 26 gauge with a 5 mm pedestal cut, and had cannula extending 1.5 mm below pedestal (#8IC315GS5SPC C315GS-5/SP). The injector properties were: 2mm depth (1.5 mm in the guide and 0.5 mm projecting out of the guide; #8IC315IS5SPC C315IS-5/SPC internal 33 gauge). The dummy properties: injector of 0.008/0.2 mm, 2 mm below pedestal to fit the 1.5 mm guide and with 0.5 mm projection below the guide (#8IC315DCS5SP C315DCS-5/SPC (SM) C315GS-5 W). The cannulas, dummies and injectors were all from Plastics One.

Dental cement procedure: Dental cement from the Ortho-Jet Package (#1334PT, langdental), was applied in either pink or transparent dental cement, following manufacturer instructions. In short, skull was covered with a thin layer of Liquid foil (#2203) for approximately one minute. Ortho-Jet liquid, in either pink or clear colors (1304) was then mixed with the Ortho-Jet powder (#1330) on aluminum foil using the Disposable brushes (#1900). Once the mixture became homogenous, it was

gently applied to the skull, making sure to partially cover the cannula pedestal. The acrylic gel was then allowed to cure for approximately two minutes, and then polished with a layer of Jet Seal (#4102).

PCR details: cDNA was synthesized from total RNA using reverse transcription PCR (RT-PCR), using the TaqMan® MicroRNA Reverse Transcription Kit (#4366596, Life Technologies) and a primer pool, per manufacturer's instructions – 5 µl of each primer were combined (total of six primers) and 470 µl of water was added in order to reach 500 µl of solution where each primer is 0.2X of the primer solution. RT-PCR was run in a 45 µl reaction containing: 18 µl RT Primer Pool, 0.9 µl dNTP with dTTP (100mM), 9 µl of Multiscribe Reverse Transcriptase (50 U/µl), 4.5 µl of 10x RT Buffer, 0.57 µl of RNAse Inhibitor (20 U/µl), 3.03 µl of water and 9 µl of 100ng/µl sample.

Following the RT-PCR, qPCR was done in triplicates of 10 µl reactions as following: 10 µl of TaqMan Universal Master Mix II, No AmpErase UNG (2x), 7 µl of Nuclease-free water, 2 µl of RT cDNA product, for a total of 40 ng cDNA input per reaction, and 1 µl of Taqman MicroRNA assays were combined. Relative miRNA expression was determined using the ΔΔCT method (Hellemans, Mortier et al. 2007), by calculating the mean difference between the cycle threshold (CT) of the miRNA of interest and the endogenous control (SnoRNA-234) for each sample (ΔCT). ΔCT for the control treatment was averaged and subtracted from the ΔCT of the treated and control samples (ΔΔCT). The fold changes for each individual sample were calculated by raising 2 to the power of -ΔΔCt (2^-ΔΔCT), and expressed as the mean fold change over the mean fold change of the control group (by dividing the treatment 2^-ΔΔCT by the averaged control 2^-ΔΔCT). Reactions were carried out in a CFX384™ Real-Time PCR Detection System (Bio-Rad).

Immunostaining details: Sections were penetrated with 0.1% Triton-X 100 (2 x 5 minutes at 25°C), washed in PBS (3 x 5 minutes at 25°C), blocked with 10% filtered goat or donkey serum (30 minutes at 25°C), and treated with 1:50 rabbit anti-GABAB-R1 (#Sc-14006-R-300, Santa Cruz Biotechnology), 1:50 rabbit anti-GluR2 (#ab52176, Abcam), 1:100 rabbit anti-GRINA (#GTX51232,

Gene Tex), 1:1000 guinea pig anti-NeuN (#ABN90, Emd Millipore), 1:500 mouse anti-NeuN (#MAB377, Neuromab), 1:300 goat anti-Iba1 (#ab5076, Abcam) antibodies (4°C overnight), washed in PBS (3 x 10 min at 25°C), and then subjected to reaction of two hours at 25°C with 1:1000 fluorescence-conjugated secondary antibodies of donkey anti-mouse Alexa 488, donkey anti rabbit Alexa 488, donkey anti-mouse Alexa 568, donkey anti-rabbit Alexa 568, donkey anti-guinea pig Alexa 660 (Invitrogen); donkey anti-guinea pig CF 405M (#SAB4600468, Sigma Aldrich), and rinsed with PBS (3 x 10 minutes at 25°C). The sections were mounted on slides using sterile 0.2% gelatin and then mounting media as applied with or without DAPI (Vector Laboratories) and cover slipped.

CHAPTER 5: DISCUSSION

The goal of my dissertation project was to investigate the role of synaptic RNAs in alcoholism. To accomplish this, I aimed to identify the mRNAs and microRNAs involved in chronic alcohol consumption, and to manipulate microRNAs in alcohol-consuming mice in order to assess the resulting behavior and composition of synaptic mRNAs. This dissertation has two particularly noteworthy findings: 1) Alcohol changes the composition of synaptic RNAs in a biologically-coordinated manner, and these changes are different than what is observed in the rest of the cell; 2) miR-411 is sufficient to alter alcohol consumption specifically, while not changing several other behaviors.

The results from this project further our understanding of the molecular underpinning of alcoholism, facilitating further research into miR-411 and microRNAs in general as the master regulators of behavior. As what frequently happens in science, in addition to advancing the field, our results have led to many new questions, and have emphasized how important and difficult it will be to reveal the answers to these. In this chapter, I will discuss our results in a more global scheme of things and address some of these questions.

"The scientific mind does not so much provide the right answers as ask the right questions."

- Claude Levi Strauss

Why Study Addiction?

The main hypothesis for this project revolves around the theme that alcoholism causes neuroadaptations through changes in genetics. By this, I refer to alcoholism as a brain disease influenced by genetics. One of the first questions people ask me when I describe my project is: why study alcoholism? Is alcoholism a disease?

The Oxford dictionary defines disease as "A disorder of structure or function in a human, animal, or plant, especially one that produces specific signs or symptoms or that affects a specific

location and is not simply a direct result of physical injury." Chapter 4 shows a clear disorder of brain cell transcript/protein composition in response to chronic consumption of alcohol, suggesting structure has changed. And all three experimental chapters show an initial escalation in consumption of alcohol and in preference for alcohol over water throughout time, as one of the many symptoms caused by alcohol. So, by brain structure definition and by symptom definition, alcoholism is a disease.

Another aspect of diseases is the rates of 1) fatality, 2) hospitalization, 3) population prevalence and 4) resources spent on the matter. A measure of alcoholism on these parameters gives further clarity of the importance of this science. (The following statistics are for the United States. It is safe to assume that these indications of importance would also stand out in other countries.) 1) The CDC shows that excessive drinking is responsible for 1 in 10 deaths among adults (Blincoe, Seay et al. 2000, Compton and Berning 2015, Jewett, Shults et al. 2015), and that approximately 45,000 people die each year from drugs with 29,000 of them caused by alcohol (excluding accidents and homicides). (As a comparator, firearms, which are also a heavy burden on American society, caused 33,636 deaths (Kochanek, Xu et al. 2011). Further, 31% of all US traffic-related deaths were due to alcohol (Jewett, Shults et al. 2015), with 19% of traffic deaths among children being caused by an alcohol-impaired driver (Control and Prevention 2012, Stahre 2014). There were nearly 4.6 million drug-related emergency room visits nationwide, with 23.5 million people needing treatment for an illicit drug or alcohol abuse problem (Substance Abuse and Mental Health Services Administration, Department of Health and Human Services, USA). 3) Alcoholism is widespread in the population, with approximately 16.3 million adults classified as alcoholics (Alcohol Facts and Statistics, National Institute on Alcohol Abuse and Alcoholism). 4) Excessive drinking costs the nation more than $249 billion annually, from costs related to crime, lost work productivity and health care (Excessive Drinking Costs U.S., CDC; Alcohol Facts and Statistics, National Institute on Alcohol Abuse and Alcoholism)(Sacks, Gonzales et al. 2015).

So it is clear that alcoholism can be fatal and has a high social and financial price-tag. The last aspect of alcoholism I would like to touch on is the loss of control. People suffering from alcoholism do so despite the negative consequences associated with the consumption of it. For example, despite broad awareness of the deadly consequences of drinking and driving, there still are 121 million self-reported episodes of alcohol-impaired driving among U.S. every year (Traffic safety facts, National Center for Statistics and Analysis), and quite likely this is an underestimate.

The next question I get asked is, if alcoholism is a disease what are the cures or treatments for it? Today, there are only three FDA approved treatments for alcoholism, and all are significantly limited due to side effects and failure to relieve drug craving. One of the main reasons that more effective therapies are not prevalent is the complexity of the disease - alcohol is caused by environmental factors and the abnormal expression of many genes. Thus, in this thesis I used novel approaches to discover these key genes in order to develop a therapy for alcoholism.

Why Study microRNAs?

In this thesis I focused on mRNAs and microRNAs in the brain. Here I will discuss why I find these specific RNA subtypes important in the brains of alcoholics. It is now well accepted that the intake of drugs of abuse can lead to fundamental changes in neuronal processes that regulate synaptic structure and function (Jin, Zarnescu et al. 2004, Klein, Lioy et al. 2007). These changes can manifest in long-lasting neuroadaptations in several key regions of the brain that may, in turn, contribute to behavioral changes characterized by altered processing of contextual information. An outstanding question that remains is: what can be responsible for the persistent drug memory that is able to linger for years and causes relapse despite all the negative consequences of alcoholism?

The DNA is stable and does not change throughout the life cycle of the cell. The molecular turnover of RNA and protein is relatively rapid, thus these molecules are not capable of encoding long-lasting effects. However, the chromatin structure of the DNA is influenced by RNAs and proteins

(epigenetics). In turn, the structure influences the downstream expression genes, and this continuous process can be perturbed by drugs of abuse. Therefore, these epigenetic mechanisms can potentially mediate the long-lasting effects of drug dependence.

MRNAs code for proteins and thereby play a role in modification of cellular epigenetics. However, microRNAs are non-protein-coding RNAs (ncRNAs). So how are they involved in this process? It is well established today that microRNAs, and ncRNAs in general, can regulate the process of translation of mRNA into protein and therefore regulate the epigenetic structure of DNA. Furthermore, protein coding RNAs only comprise 2% of the genome, while at least 80% of transcripts floating in the cell are non-coding (Djebali, Davis et al. 2012). As the cell transcribes a lot of these ncRNAs, they must play an essential role in cellular function. This view is in stark contrast with what was commonly believed not very long ago: ncRNA were considered largely as "junk". (Science sometimes struggles at dealing with the unknown. This "junk" classification was so far from the truth, it sounds today almost like the believing the world was flat.)

Interestingly, while the proportion of the protein coding genes remains relatively static across organisms, analysis of ncRNA complexity through evolution reveals that the proportion of non-coding sequences in the genomes highly correlates with the complexity of the organism (Barrett, Fletcher et al. 2012), suggesting that regulation of the gene expression is increasingly important with higher level organisms.

Complex organisms have a more complex brain and exhibit more complex behaviors suggesting the brain may be a particularly prominent organ within which microRNAs play an important role in controlling gene expression. Hence, it is likely that microRNAs play a critical role in basic aspects of brain function and behavior. Data from human alcoholics show changes in the expression of microRNAs in the brain, suggesting they play a role in alcohol related behaviors (Lewohl, Nunez et al. 2011).

However, the specific role of microRNAs in the brain is still widely unknown and the exact molecular mechanisms governing microRNA functionality are far from clear and are the subject of intense scientific inquiry. Consequently, the data presented in this dissertation aimed at revealing a neurobiological mechanism through which microRNAs are involved in regulation of mRNAs in regards to alcohol.

To gain an understanding into the role of microRNAs in alcoholism, I started by investigating alcohol-induced mRNA expression in the brain (chapter 2), with the hypothesis that if microRNAs regulate mRNAs, then by drawing a map of mRNAs involved, I would be able to predict which microRNAs would be involved. Results revealed that many of the mRNAs which were responsive to alcohol were part of the same biological pathways, further emphasizing the overarching effects of alcohol on the brain. I then studied the brain microRNAs which were altered by alcohol (chapter 3) and discovered certain alcohol-responsive microRNAs which were predicted to target the alcohol-responsive mRNAs from the same biological pathways, suggesting functional importance for the microRNAs. Lastly, I manipulated five of these microRNAs in the brain in order to affect alcohol consumption (chapter 4). Results from these experiments highlight some of the roles of microRNAs in alcohol-related behaviors Future research is needed in order to elucidate the molecular mechanisms through which this occurs.

Why and How do the RNAs in the Synapse Respond Differently to Alcohol from those in the Soma?

To add complexity to this story, it is well established that the specific mRNAs and microRNAs in particular cellular compartments play different molecular roles, but it is unclear what roles and why. In this project, I attempted to answer this question by profiling the mRNAs and microRNAs from specific cellular compartments and discovering their role in alcohol consumption.

While most mRNAs are restricted to the neuronal soma, significant amounts of mRNA are found in synaptic compartments of the cell and translated into protein locally (Steward and Levy 1982,

Steward and Banker 1992). It stands to reason that molecules in the vicinity of the synapse may be the ones involved in the process synaptic plasticity. Studies examined the composition of microRNAs in the synapse and revealed many synaptically enriched and depleted microRNA families (Lugli, Torvik et al. 2008, Eipper-Mains, Kiraly et al. 2011, Pichardo-Casas, Goff et al. 2012). RNAs – specifically in the synaptic compartments of the cell – are found to play a key role in the different states of addiction, such as dependence, tolerance, withdrawal and craving (Russo, Dietz et al. 2010, Eipper-Mains, Kiraly et al. 2011, Mayfield and Nunez 2012). Therefore, I aimed to identify the molecules in the vicinity of the synapse which are involved in chronic alcohol consumption. To do this, I profiled mRNAs and microRNAs from SNs and paired TH preparations from the amygdala of mice chronically consuming alcohol. Using a within-subject comparison, I found a robust difference between the alcohol-responsive mRNAs and microRNAs detected in the SN and TH. However, the question remains as to why there is a difference between the composition of RNAs in the SN and TH. How is this mechanistically possible? First, by using the SN preparation, I restricted the expression profiling to the synaptic compartments, thus preventing the dilution of the synaptic RNAs with the somatic transcriptome. The response of RNAs to alcohol in the synapse may be of larger magnitude than that seen in the soma, but this would become diluted as RNA is extracted from total, unfractionated tissue. Second, alcohol could selectively target synaptic RNAs, ultimately changing gene expression in the synapse, and this could be different or even opposite from the response in the TH. If the magnitude of treatment fold changes for the same RNAs were larger in SN compared with TH, it would suggest that synaptic enrichment/dilution was responsible for the differences seen between SN and TH response to alcohol. However, results presented in chapter 2 show that only some mRNAs had a similar response to alcohol, whereas some mRNAs responded in complete opposite directions. Furthermore, results reported in chapter 3 indicate that only one microRNA, (out of 1,111 microRNAs tested), miR-411, responded in a similar direction in both SN and TH, suggesting even more so that the differences between SN and TH are due to localized effects of alcohol.

How is Alcohol Affecting these Cellular Compartments Differently?

One explanation to this would be that alcohol may be affecting the processing of microRNAs into their mature form, specifically in the synapse, allowing for the detection of these microRNAs (on our microRNA arrays) and rendering them active/inactive to regulate synaptic mRNA expression. In chapter 2, I showed that alcohol changed the expression of synaptic Dicer and eif2c (also known as Ago2). It stands to reason therefore that alcohol changes microRNA composition through microRNA processing enzymes, ultimately changing mRNA composition in the synapse. This is supported by data from Lugli et al., who have demonstrated that microRNA precursors are present in the synapse as well as the microRNA processing enzymes, and that their activity is modulated by neuronal activity (Lugli, Torvik et al. 2008). Furthermore, in chapter 3, I revealed that synaptic miR-92a, miR-92a-1∗, miR-92a-2∗, and miR-92b, microRNAs are derived from the same premature precursor or derived from the 5' and 3' ends of the same double strand and were all upregulated in response to alcohol. Moreover, the precursor for these microRNAs, Pre-miR-92a (unpublished data), was also upregulated in response to alcohol - highly suggesting that the premature form of the microRNA was processed into the mature form in response to alcohol. This was not seen in the TH, suggesting these processes occurred in the vicinity of the synapse. Interestingly, miR-92b is involved in synaptic signaling (Ceman and Saugstad 2011) and may be involved in the aberrant synaptic plasticity seen after alcohol exposure.

A second hypothesis which can explain the different responses to alcohol between the SN and TH would be that alcohol affects the synaptic translation of mRNAs by binding to the translational machinery, and these in turn affect the levels of microRNAs. There are two ways changes in mRNA expression can affect microRNA levels – first, if the mRNA encodes for a protein that regulates the transcription of the microRNA (such as a transcription factor). This has shown to be the case with miR-212 and MeCP2 where the levels of MeCP2 affect its association with the DNA encoding for miR-212, thus affecting levels of miR-212 (Im, Hollander et al. 2010). In chapter 3, I described how alcohol

affects the expression of the synaptic translational machinery and the expression of a variety of transcription factors suggesting this may be the case. Furthermore, I identified ten alcohol-responsive synaptic microRNAs that were all located in nearby areas of chromosome 12 and were all downregulated by alcohol, suggesting that the observed expression changes by alcohol resulted from modification on a transcriptional level. This further supports the notion that alcohol directly affects mRNAs and those in turn change the transcriptome in downstream expression of microRNAs. A second way by which the effects of alcohol on mRNAs affect microRNA expression downstream is when mRNA levels change, it changes the number of available targets for microRNAs to bind to, causing these microRNA to float solo in the cell leaving them exposed to degradation, or allowing them to bind to other mRNA targets which they would not have been binding otherwise. Data from studies examining RNA processing enzymes suggest that change in the composition of RNA in the cells can change the probability of these interactions. Moreover, when I manipulated miR-411 (chapter 4), alcohol consumption and preference was decreased in alcohol-consuming mice. However, when I gave the same treatment to alcohol-naïve mice, there was no difference in subsequent consumption levels between the miR-411 manipulated group and control. Thus, miR-411 does not influence behavior in a normal mouse, but does influence behavior after alcohol-induced neuroadaptations take place. This suggests that alcohol directly influences mRNA expression (specifically mRNAs targeted by miR-411), and that the cellular response to that is to change the levels of miR-411 in order to oppose the change in mRNA levels and bring the cell back to homeostasis (Nunez, Truitt et al. 2013), further supporting the idea that alcohol targets mRNAs which in turn affect microRNAs.

Lastly, the differences between the response to alcohol in SN and TH may be caused by the effects of alcohol on the trafficking of RNAs to the synapse, resulting in unique SN-enriched RNAs. Whether alcohol directly affects the activity of the synaptic microRNA processing machinery, the activity of the mRNA translational machinery, or the trafficking of RNAs to the synapse, may be tested using an enzyme activity assay with the application of alcohol. One, two, or all three of these cases

may be true (alcohol specifically affects synaptic microRNA processing, synaptic mRNAs directly or translation of mRNAs, or trafficking of RNAs to the synapse), and it is exciting to take part in the science that will lead to an answer to this question. The SN studies help pinpoint the role of synapse-related microRNA-mRNA interactions and demonstrate the importance of the discrete cellular microenvironment in identifying the effects of alcohol.

Studies of local translation can be aided by new tools to block translation of specific genes of interest in specific areas such as axons (Lin and Holt 2007), using microfluidic compartmentalized cultures (Coquinco and Cynader 2015, Jain and Gillette 2015) and axonal application of siRNA (Cox, Hengst et al. 2008). Application of these and other refined tools will advance our appreciation of localized control of gene regulation orchestrated by microRNA and mRNA populations. The activity of individual microRNAs in discrete cellular compartments underscores their essential role in cellular function and the widespread impact that drugs of abuse can exert by targeting microRNAs.

How is it that Synaptic microRNA–mRNA Interactions Respond to Alcohol in a Coordinated Manner?

Experiments in chapter 2 revealed that many of the synaptic mRNAs responsive to alcohol were from similar biological pathways and had a similar change in expression (co-expression) in response to alcohol. A question arises as to how these mRNAs know when to respond to alcohol. And how do they communicate to respond in a coordinated manner?

It is reasonable to believe that a common regulator, such as a microRNA, can regulate the co-expression of these mRNAs in a coordinated manner. Studies using computational sequence analysis predicts that a single microRNA can target 10–100s of mRNA transcripts (He and Hannon 2004), and these may be alcohol-responsive mRNAs (Lewohl, Nunez et al. 2011, Tapocik, Solomon et al. 2013). In addition, each mRNA transcript can be targeted by potentially hundreds of miRNAs. In chapter 3, I show that a subset of alcohol-responsive microRNAs were predicted to target many alcohol-responsive mRNAs in the synapse, and that these may be participants of the same biological pathway.

For example, I found that synaptic miR-369∗, tumor necrosis factor α (TNF-α), fragile-X mental retardation-related protein 1 (FXR1) and argonaute 2 (Ago2) were altered in response to alcohol. Other studies have shown that miR-369* directly associates with mRNA to initiate its activation under conditions of arrested growth (Vasudevan, Tong et al. 2007), and this effect is dependent on the recruitment of the RNA-binding proteins (Vasudevan, Tong et al. 2007). These results are a demonstration that all of the vital components of this pathway were coordinately changed in SN, lending support to the utility of the SN preparation in studying alcohol regulation of the transcriptome.

Also in chapter 3, I utilized a combination of unbiased network and bioinformatic methods to identify microRNAs and mRNAs with overlapping patterns of expression that correlated with alcohol consumption. I also identified the biological pathways associated with those mRNAs, pinpointing the alcohol-responsive microRNA which may regulate certain mRNAs and result in change in specific biological pathways. The co-expression of microRNAs with a network of alcohol-responsive mRNAs further supports the role of microRNAs as master regulators of translation in the synapse.

The co-expression of microRNA also illustrates a mechanism by which alcohol changes the expression of a few microRNAs and how they can cooperate to target an mRNA that is known to be involved in alcoholism as well. This cooperation between the microRNAs may be of particular importance because it enables a reduced number of active microRNAs to regulate gene expression, greatly impacting synaptic RNA composition and the response to alcohol. Because alcoholism is a complex trait with global changes in gene expression, microRNAs serve as worthy targets for treatment, as they control global cellular changes in gene expression. Further studies will be needed to validate the individual interactions between microRNAs and mRNAs. Nevertheless, the combined approaches provide a list of potential alcohol-sensitive interactions in the synapse that are candidates for further investigation.

Can microRNAs be used as Targeted Therapies for Human Diseases?

In the last chapter of this thesis, I hypothesized that manipulation of microRNA levels in the opposite direction produced by chronic alcohol consumption should oppose the neuroadaptations caused by alcohol, and reduce alcohol consumption. The rationale of using microRNAs as an anti-addiction therapy was the result of the observation that microRNA expression is altered in addiction compared with healthy 'normal' samples and that microRNAs are capable of targeting many genes from the same cellular pathways and functions, enabling the use of a small number of microRNAs to achieve an orchestrated regulation of a cellular pathway.

There are three approaches for manipulating an expression of a microRNA: 1) Directly targeting the microRNA with specific nucleotide constructs - such as the microRNA antagomiRs and mimics (such as the ones I have used in chapter 4). 2) Using viral vectors to deliver the DNA encoding the microRNA/microRNA inhibitor into the genome. 3) Using a manipulator that can affect a microRNA (either directly or indirectly) while also affecting other molecules. I explain each of these approaches below:

1) In chapter 4, I showed that antagomiR-411 was successful in decreasing alcohol consumption and preference in alcohol-consuming mice without affecting total fluid intake or saccharin consumption. Future studies will need to address whether this treatment can be administered safely to humans and whether it is successful in altering behavior.

There is however evidence for antagomiRs being safe to use in humans and having clinical importance. AntagomiR-122 was safely tolerated in chimpanzees and healthy humans (van Rooij and Kauppinen 2014), and was successful in treating chronic hepatitic C virus (HCV) infection in mice (Baek, Kang et al. 2014). Another clinical trial used antagomiR-34, encapsulated in a liposomal nanoparticle formulation (MRX34) to inhibit the formation of cancer stem cells by inhibiting multiple oncogenic pathways as well as stimulate anti-tumor immune response to induce cancer cell death (Bouchie 2013). In Phase 1 trial, MRX34 has demonstrated partial responses to treatment in patients

with renal cell carcinoma, acral melanoma and hepatocellular carcinoma. The evidence for the clinical efficacy and tolerability in humans for microRNA therapeutics in treating cancer suggests that this may be a valid form of treatment for other diseases such as addiction. Future studies are warranted in order to identify and test treatments in humans suffering from addiction.

2) The second way to manipulate microRNAs can be done with the use of viral vectors which incorporate these inhibitors and activators into the DNA under certain promoters, such that the cell itself will be synthesizing them. The vectors available today can be activated by simple consumption of a drug and can also be halted in a similar manner. The ability to both activate and stop a specific microRNA from being transcribed is of importance - It enables the long-lasting expression of the treatment while providing a possible cessation of a treatment in case it causes severe side effects. This also enables an elegant within subject analysis, where it is possible to test the changes in behavior with and without that microRNA in the same organism.

3) The third method is to manipulate microRNA expression through the use of drugs to modulate microRNA expression by targeting their synthesis, transcription and their processing (Ling, Fabbri et al. 2013, Giza, Vasilescu et al. 2014) or by the use of vagal nerve stimulation (Jiang, Li et al. 2015). In chapter 3, I analyzed the differentially expressed mRNAs and microRNA as potential targets for known drugs and identified ten drugs. Seven of these ten drugs are FDA-approved, with several of these having known links with alcohol actions. The FDA approved drugs provide a potential off-label treatment for alcoholism in humans. Because these are approved for use in humans with known side effects, this may allow foregoing the animal studies and proceeding directly to human studies.

Currently, there is an ongoing clinical study to assess the safety and efficacy of gemfibrozil in modulating microRNA-107 levels for the prevention of Alzheimer's disease in subjects with intact cognition and mild cognitive impairment (clinicaltrials.gov identifier # NCT02045056). Another clinical trial (which is opening soon) is set to determine the effects of transcutaneous vagal nerve stimulation on plasma microRNAs in healthy humans with the hope of using this to treat epilepsy in the future

(clinicaltrials.gov identifier #NCT02359188). Further evidence from rats demonstrates that miR-210 mediates the vagus nerve stimulation-induced neuroprotection following cerebral ischemia/reperfusion injury (Jiang, Li et al. 2015).

These are exciting times to be a researcher in the microRNA field, as the role of microRNAs in the brain and their relationship to behavior is being elucidated and their direct therapeutic efficacy in humans is being revealed. Call me optimistic, but I believe that soon enough we will be able to find safe and efficacious ways to treat brain diseases with microRNA manipulators. Though since microRNAs are master regulators of gene expression in the cell, and since there are roughly 25,000 genes in a human cell, it may take time to reveal the exact molecular mechanism by which microRNA exert their effects. It is my hope that continued scientific activity in this field will overcome these odds and help find a pathway to alcohol related therapies.

"Science never solves a problem without creating ten more."

- George Bernard Shaw

Future Directions

MANIPULATION OF SYNAPTIC SPECIFIC MICRORNAS

In order to attempt changing alcohol consumption levels, I chose to manipulate five key alcohol-responsive microRNAs in the PFC of mice: miR-411, miR-203, miR-92a, miR-187 and miR-137. In chapter 4, I showed that with the exception of miR-411, the manipulation of these other four microRNAs did not change alcohol consumption levels or preference, whereas manipulating miR-411 did manage to change consumption levels and preference significantly. In chapter 2, chronic alcohol consumption was found to change the expression of these microRNAs (miR-411, miR-203, miR-92a, miR-187 and miR-137) in the synaptic areas of the cell, and that this effect can be different from that seen when measuring the whole-cell total homogenate alcohol-induced expression of these microRNAs. This is true for all of these microRNAs except for miR-411, which was decreased by alcohol

both in synaptic regions of the cell as well as the whole-cell total homogenates. These results imply that it may be necessary to manipulate a microRNA that is influenced by alcohol in the whole cell and not just in the synapse, and that a microRNA that is only affected by alcohol in the synapse may have different or opposite effects in the rest of the cell (referred to as total homogenates). However, this explanation is unlikely since alcohol consumption and dependence was found to change the expression of these microRNAs in the total homogenates of the PFC as identified by other studies in humans - miR-203 and miR-92a (Lewohl, Nunez et al. 2011); in rats - miR-187 and miR-137 (Tapocik, Solomon et al. 2013); in mice - miR-187 (unpublished data), and in non-PFC cell cultures - miR-203 (Van Steenwyk, Janeczek et al. 2013) . One way to test this would be to manipulate the microRNAs that are changed by alcohol in the total homogenates but not in the synaptic areas. A more direct way to answer this could be to manipulate these microRNAs in a spatially specific location in the cell using viral vectors that express microRNAs specifically in the synapse or in the soma and compare the behavioral changes.

Another explanation to as why only one of five of our microRNAs was successful in changing behavior is that different microRNAs are involved in various periods of alcohol dependence, such that different time points are necessary for the manipulation of these microRNAs to take an effect. One example is from Im et al. who increased the levels of miR-212 and demonstrated that cocaine intake is markedly lower in miR-212 rats with extended access to cocaine, but not in rats with restricted access to cocaine (Im, Hollander et al. 2010).

MANIPULATION OF MICRORNAS WITH A RESCUE

One of the consensus experiments in this scientific field is to show that a certain molecule is both *necessary* and *sufficient* to explain a change in behavior. In order to do that, some studies include a 'rescue experiment', where they use one type of manipulation to prove it is sufficient in changing a behavior, and then use the opposite manipulation to show that the induced-behavior is stopped when

taking away the original manipulator, thus determining the necessity of the molecular manipulation. In chapter 4, I conveyed that antagomiR-411 was sufficient to decrease alcohol consumption and preference while reducing the levels of miR-411. Unfortunately, when increasing the levels of miR-411, I did not see the opposite behavior – when using mimic-411 there was no increase in alcohol consumption and preference. Because of this issue, I was not able to do a rescue experiment. Future studies will need to find a good opposite treatment to antagomiR-411 in order to set up a rescue experiment. One way to find a treatment that increases alcohol consumption would be to do a dose response curve with mimic-411 and measure the changes in alcohol consumption in each dose. Another possibility would be to use brain specific viral vectors to deliver miR-411 under an active promoter which would chronically drive up the levels of miR-411.

There is a current effort of the W.M. Keck Center for non-coding RNAs (University of California, San Francisco) together with the Jackson laboratories (Bar Harbor, Maine), to create transgenic mice with genetic deletion of individual microRNAs. They also produced a strain that has the option to both induce the knockout and then later remove the knockout to restore a functional wild type allele. Performing this step is critical for a rescue experiment, allowing the measurements of the before and after for the knockout in the same mouse (Park, Jeker et al. 2012)(http://rna.keck.ucsf.edu/miRKO-DB).

Another way to show that a microRNA is both sufficient and necessary for the aberrant translation of proteins during addiction would be to halt the translation process during the manipulation of the addiction-behavior. Some antibiotics (such as anisomycin) have the ability to inhibit protein translation (Chan, Khan et al. 2004). If indeed microRNAs control mRNA translation in addiction without an intact process of protein translation, a microRNA manipulation will not be able to affect addiction-related behaviors.

Conclusion

The one pervasive theme throughout this dissertation is alcohol's propensity for modifying the neural landscape which is reflected by its many responsive molecules and signaling pathways. Establishing causality between alcohol and the succeeding cellular and behavioral adaptation is a difficult task, and identifying the small effects on each system that culminate in alcoholism is a complex challenge. Evidence gathered through pharmacological manipulation, genetic modification, and direct measurement supports the importance of microRNAs in alcohol-induced neuroadaptations. The availability of a large variety of animal models (ranging from flies and worms to primates), as well as the advancement of neurobiological techniques (genomics, transcriptomics, proteomics, function-omics) provide hope for discovering the molecular mechanisms underpinning the neurobiology of alcoholism and revealing new therapeutic targets for this societal health burden. If microRNAs live up to their role as master regulators, then their impact on drug-mediated responses and therapeutic strategies will be of critical clinical importance. The results of this thesis project provide proof of concept that synaptic gene networks can yield viable candidates for manipulation of behavior, and provide fertile ground for the development of treatments for alcohol use disorders.

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VITA

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